Developing a blood glucose meter-based method for the rapid detection of lactose in dairy ingredients.

by

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B.S., University of Wisconsin-La Crosse, 2015

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

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Abstract

Lactose is an important nutritional, functional, and economical constituent of dairy ingredients. The lactose content of a dairy ingredient can often be directly associated with that ingredients value, shelf life, and finished application use. Lactose assays commonly employed by the dairy ingredients industry are time-consuming and require sophisticated equipment or trained staff to perform. A blood glucose meter (BGM) biosensor based lactose analysis has been developed for milk, but has not been documented for proper use with more compositionally variable systems such as dairy ingredients. The proposed BGM lactose analysis method involves diluting a sample to an appropriate level with buffer, adding lactase enzyme, incubating at 40°C for 15 minutes, and measuring the resulting glucose content with the BGM. A calibration curve developed between the known lactose concentration and BGM reading is used to quantify lactose in unknown samples. The objective of this study was to evaluate the BGM lactose analysis method in whey- and skim milk-derived (WD and SMD, respectively) ingredients. Measurement interferents such as solution pH and different types or amounts of non-lactose solids present in the measurement background were investigated for their impact on BGM output bias; it was found that measurement solution pH must be between 6.7 -7.0, and that calibration procedures must be designed to correct for large differences in non-lactose solids that may be present when measuring different materials. A standardized calibration procedure was then developed and verified by measuring the lactose content of model WD and SMD ingredient dilutions using the proposed BGM method and an enzymatic spectrophotometric absorbance (EZA) reference method. Lactose in 15 dried commercial dairy ingredients of both WD and SMD type over a broad range of lactose contents (0.01-81.9% lactose as-is) was then quantified using the BGM method and EZA reference method. When the correct calibration procedure and an optimal

BGM-brand are employed, excellent BGM assay accuracy was obtained for the commercial samples, with an average absolute percent bias difference of <5% noted between the BGM method and EZA reference method results. Furthermore, an average percent coefficient of variation of <3% between duplicate measurements for the BGM method in these commercial samples indicates excellent method precision. Overall, the BGM method is a promising tool for rapid, low-cost analysis of lactose in dairy ingredients for purposes such as quality control, process development, or ingredient standardization.

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Acknowledgements

I would like to formally acknowledge Dr. Jay Amamcharla for taking on the extra time and responsibility that comes with mentoring a distance graduate student through a thesis MS program. I would also like to thank Drs. Anand Rao and Karen Schmidt for serving on my graduate committee. My friends in both Dr. Jay's lab and Agropur's protein research center all deserve a warm thank you for their help with ordering supplies, arranging testing, and making time in their busy schedules for me without hesitation. Lastly, I want to acknowledge both Agropur Ingredients (La Crosse, WI) and Midwest Dairy Foods Research Center (St. Paul, MN) for their respective tuition and research financial support.

Dedication

I would like to dedicate this work to my wife, parents, friends, co-workers, and cats for providing the excellent support system that they do. Some part of this is also owed to Jill Rippe, for pushing me early on in my career to be creative and always take the path less traveled.

Chapter 1 - Introduction

The dairy industry lacks a simple and rapid way to quantify lactose. Most conventional assays for lactose are slow, expensive, and require specialized equipment or trained personnel. Biosensors offer an attractive alternative to these traditional techniques due to their specificity, simplicity, expediency, and potential portability. However, few commercial biosensors specific to lactose exist because dairy foods and ingredients are very complex matrices for biosensors to operate in.

Blood glucose meter (BGM) biosensors may hold promise as rapid, cost-effective tools to quantify lactose. An accurate BGM-based lactose assay has already been developed for milk; the method was shown to be rapid (<15 mins) and low cost (< $0.50 per measurement). In the BGM method, milk was diluted to adjust the lactose to a measurable range, and β-galactosidase was used to enzymatically convert lactose into the glucose substrate that can be measured by the meters. While the BGM output should theoretically be one-half the w/w concentration of lactose in solution, it has been shown that calibration of the BGM output is necessary to convert the meter readings from glucose into percent lactose.

The BGM-based lactose analysis is very promising given its very low-cost and wide commercial availability relative to other biosensor-based lactose analyses. However, milk is but one dairy food out of many; a natural extension of the work done in milk is to apply the BGM lactose assay concept to dairy ingredients, which offer unique challenges compared to milk due to their variable composition.

Chapter 2 - Literature Review

A basic understanding of lactose's properties and its role in dairy ingredients is required to justify the importance of a rapid, low cost lactose analysis using blood glucose meter (BGM) biosensors. Knowledge of how dairy ingredients are made and the range of ingredient compositions that can be expected is necessary to establish practical operating procedures for a BGM lactose assay specific for dairy ingredients. Lastly, a survey of documented interferents affecting BGM accuracy is necessary to guide the investigation into what will be important in developing a robust BGM-based lactose assay for dairy ingredients.

Overview of Lactose

Lactose is the principle carbohydrate found in mammalian milk, composing 4.5%- 4.8% of cow or caprine milk (Huber and BeMiller, 2017). The amount of lactose in milk is directly proportional to the fat, casein, and ash content of the milk regardless of animal species (McSweeney and Fox, 2009); the factors that impact the relative proportions of these components in milk include the animal's environment, lactation cycle timing (McSweeney and Fox, 2009), and overall health (Booth et al., 2017).

Lactose is an economically and nutritionally important constituent of dairy products. Lactose, being a large proportion of total solids and a contributor to the sensory quality of fluid milk, has a large economic impact in terms of raw milk value (Lynch and Barbano, 2007). Formulating adequate amounts of lactose into infant nutrition products is critical for balanced nutritional delivery (Paterson and Kellam, 2009). Lactose has been shown to increase mineral adsorption and to also have a positive impact on the growth of prebiotic enteric bacteria (Dirienzo, 2016). While the presence of lactose can be beneficial, it can also be undesirable: Lactose presence can lead to undesirable defects in dried dairy ingredients and foods like frozen

desserts if these products are not processed and handled properly (McSweeney and Fox, 2009). Lactose intolerance affects the majority of the human population (McSweeney and Fox, 2009), and recent studies have indicated that formulating lactose-free dairy products may be in the dairy industry's best interest (McCarthy et al., 2017).

Structural properties of lactose.

The disaccharide lactose consists of one D-glucose and one D-galactose units each in a 6-carbon cyclic hemiacetal ring conformation connected by a β-1,4 glycoside bond (Durham, 2009). While lactose is found primarily in free disaccharide form, small amounts naturally occur polymerized into higher oligosaccharides (Huber and BeMiller, 2017).

Lactose is not readily digested by adult humans due to the unique β-1,4 linkage between the glucose and galactose units. Mammalian infant digestion systems (humans included) naturally manufacture the enzyme (β-galactosidase) to digest lactose via hydrolysis of the β-1,4 glycoside bond, but this ability usually goes away for most species upon weaning from a mother's milk; discrete subpopulations of humans descending largely from north-western Europe and the northern portion of the Indian sub-continent are unique exceptions to this rule. (McSweeney and Fox, 2009).

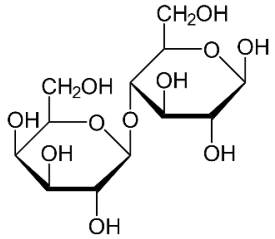


Figure 2-1 Chemical structure of β-lactose. Image courtesy of wiki commons (public domain).

Since lactose cannot usually be completely removed from dairy products by any commercial physical means, the production of lactose-free dairy products generally involves the

enzymatic *ex-situ* hydrolysis of lactose's β-1,4 glycoside bond using manufactured β-galactosidase enzyme (commonly referred to as lactase). This results in separate D-glucose and D-galactose constituents for every lactose molecule hydrolyzed as illustrated in figure 2-2. While the process can be costly, lactose-free dairy products are consumable by lactose intolerant individuals, providing a huge growth opportunity for the dairy industry (Horner et al., 2011; McCarthy et al., 2017).

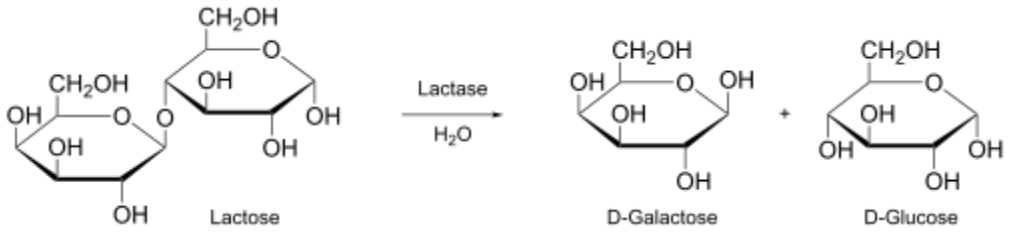


Figure 2-2 Lactose hydrolysis via lactase enzyme. Image courtesy of wiki commons (public domain)

Chemical properties of lactose

The free aldehyde group located on the D-glucose unit in lactose contributes to much of the unique chemistry associated with lactose. For one, this free aldehyde group allows lactose to participate in redox reactions such as Maillard browning, making lactose a reducing sugar (Durham, 2009). The carbon in this same group is also chiral, allowing lactose to exist in 2 distinct anomeric forms referred to as α- or β-lactose (Wong and Hartel, 2014). When in solution, repeated conversion of the glucose moiety between closed-cyclic and open-chain forms allows lactose to switch between the α and β form in a dynamic equilibrium process known as mutarotation (Durham, 2009; Wong and Hartel, 2014; Huppertz and Gazi, 2016).

The equilibrium concentrations of α– and β -lactose established by mutarotation in solution can be manipulated by factors including temperature and total lactose concentration (Durham, 2009; Huppertz and Gazi, 2016). Temperature especially is used to determine which

form of lactose will be formed during industrial crystallization processes, with the α form being favored at temperatures < 93.5 C°, and β being favored at temperatures > 93.5 C° (McSweeney and Fox, 2009).

The rate of lactose mutarotation is affected by pH, temperature, and ionic concentration: The minimum mutarotation rate is found at pH 5, while the rate is maximized when pH is < 2 or >7. Every 10 C° increase in temperature raises the mutarotation rate ~ 2.8 times. The presence of milk salts has been found to increase the rate by ~1.8 -2.2 times (Durham, 2009; Huppertz and Gazi, 2016).

Physical properties of lactose.

Some physical properties of lactose in water at 20 C° differ significantly depending on the anomeric form of the lactose being studied. For example, solubility can range from 70 g/L for pure α – lactose and 500 g/L for pure β-lactose, and the two forms can easily be distinguished by their specific rotations of +89° or +34° for α- or β-lactose respectively (McSweeney and Fox, 2009).

Purified lactose is usually stored in dried crystalline form, with the shape and storage characteristics of these crystals being dependent on the pre-drying anomeric form and crystallization extent. α-lactose crystals are usually in hydrate form and have a distinctive tomahawk shape; they are very hard, stable, and slow to dissolve. β-lactose generally crystallizes in an uneven diamond shape (Wong and Hartel, 2014), and have improved dissolution and solubility characteristics over α-lactose crystals. A third crystal form, glassy amorphous lactose, is formed when lactose- containing products are dried too quickly (Durham, 2009) or when inadequate lactose pre-crystallization is applied before drying of high lactose ingredients (McSweeney and Fox, 2009); this unstable mixture of α-and β – lactose is very

hygroscopic and will cause caking and clumping of the powder if not handled and stored properly (McSweeney and Fox, 2009). The hygroscopicity of amorphous lactose is largely due to viscous flow and subsequent agglomeration of the glassy amorphous lactose particles at temperature and humidity levels above the glass transition phase of the material (Huppertz and Gazi, 2016).

Overview of Dairy Ingredients

Dairy ingredients are diverse in terms of their intended applications, sources, compositions, and manufacturing styles. While dairy ingredients can be used in their liquid form when convenient and economical, they are more often used in powdered form since that allows more convenient storage and improved shelf life qualities (Schuck, 2009). Some of the most common ingredients include skim milk powder (SMP), sweet whey (SW), whey protein concentrate (WPC) or isolate (WPI), milk protein concentrate (MPC) or isolate (MPI), caseinates and whey or milk permeate.

Lactose is present in most dairy ingredients, comprising <2% of dry protein isolates to 100% of pure lactose powders (Huppertz and Gazi, 2016). The amount of lactose in an ingredient is generally inversely proportional to the ingredient's protein content. Figure 2-3 conveys how compositionally diverse dairy ingredients can be.

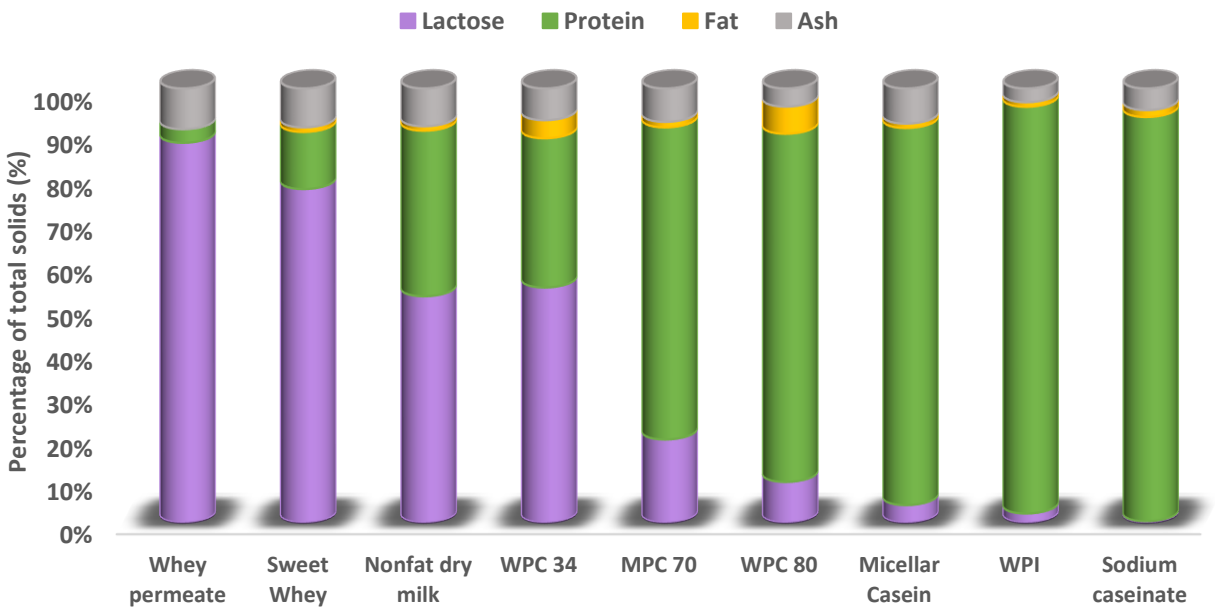


Figure 2-3 Composition of some common dairy ingredients. Adapted from the Wisconsin Center for Dairy research, Dried Dairy Ingredients, 2nd Ed. January 1st, 2017

Manufacturing of dairy ingredients

Dairy ingredients are derived from liquid dairy streams, primarily fluid milk and cheese whey, but occasionally greek yogurt whey and acid-casein curd or whey (Huppertz and Gazi, 2016). Some ingredients, such as SMP, SW, or permeates are dried directly without any further processing other than lactose pre-crystallization and possibly protein content standardization. However, in order to increase their economic value, most ingredients are processed further to isolate specific components or to increase the relative proportion of protein in the final ingredient. In either case, the core principle is to remove fat, lactose, and minerals to confer some functional or nutritional benefit to the ingredient. A brief description of the most commonly encountered dairy ingredients and how they are made is given here to understand why there is so much diversity in the dairy ingredient category.

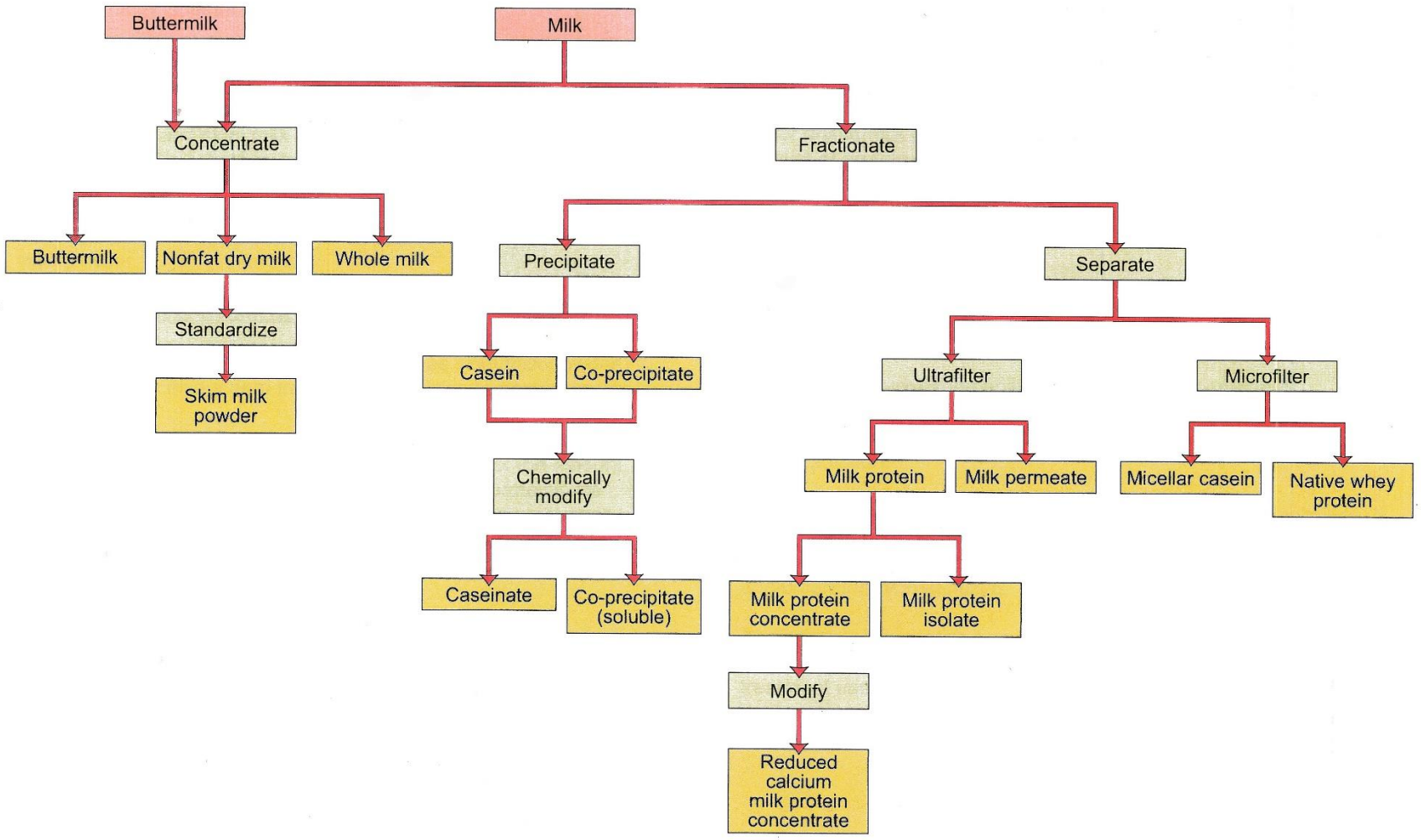


Figure 2-4 General processing schematic of milk derived dairy ingredients. Image courtesy of the Wisconsin Center for Dairy research, Dried Dairy Ingredients, 2nd Ed. January 1st, 2017, and is used with permission (personal communication).

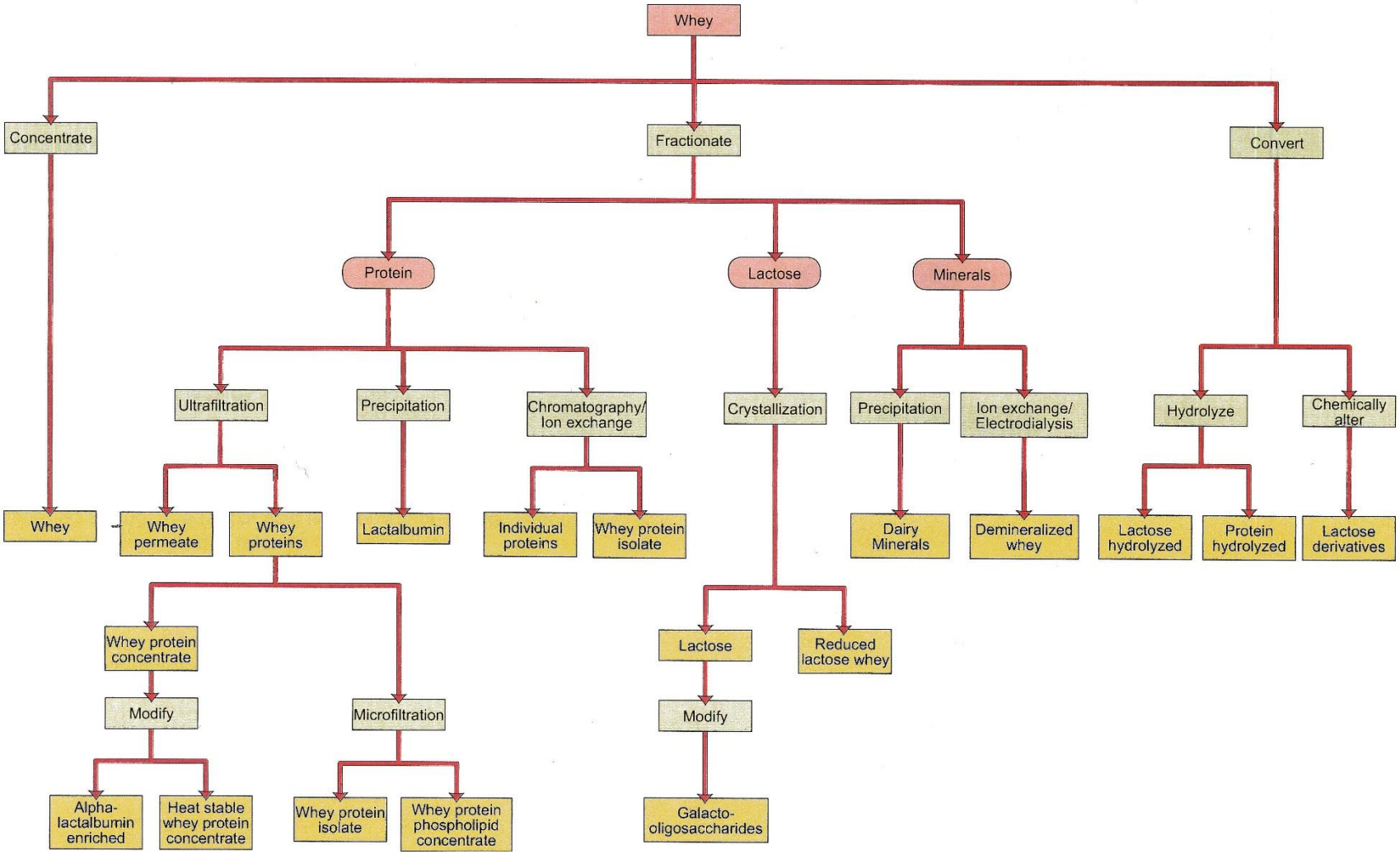


Figure 2-5 General processing schematic of whey derived dairy ingredients. Image courtesy of the Wisconsin Center for Dairy research, Dried Dairy Ingredients, 2nd Ed. January 1st, 2017, and is used with permission (personal communication).

Production and uses of MPC & MPI ingredients

Dried MPC and MPI ingredients come in an approximate dry-basis protein range of 42% - 85%, with the protein composition often resembling the original casein: whey ratio of about 80:20 found in milk (O'Kennedy, 2011). The ingredients are usually prepared via membrane ultrafiltration of skim milk, with the higher protein materials also requiring diafiltrating (Agarwal et al., 2015). The membrane filtration process allows portions of lactose, soluble minerals, and some nonprotein-nitrogen compounds to pass through the membrane in the "permeate", leaving micellar casein, whey proteins, micellar mineral complexes, and residual fat behind in the "retentate" in their original relative proportions (Agarwal et al., 2015). The membrane filtration process usually preserves the micellar nature of casein proteins in MPC and MPI products, which contributes to the ingredient's unique and desirable properties in a variety of applications: It is common practice to use MPC in cheese-milk protein standardization, improving yields and product quality. MPC and MPI are commonly used in frozen desserts and beverages to increase protein content without adding extra lactose. MPC works well to enrich the protein content of yogurt pre-fermentation, leading to improvements in product texture and nutrition (Agarwal et al., 2015).

Production and uses of caseinates

The production of caseinate requires the acid-precipitation of micellar casein from milk as a pre-requisite. The insoluble acid casein subsequently undergoes alkalization using strong caustics such as sodium, potassium, or calcium hydroxide in order to bring the material back to a soluble state (O'Kennedy, 2011); the name of a caseinate (e.g. sodium caseinate) reflects the cation present in the caustic used for this step. These ingredients differ significantly from their native micellar forms in terms of their functionality and are very efficient emulsifiers in

applications such as coffee whiteners and cream liqueurs (O'Kennedy, 2011). Due to the separation efficiency of the acid precipitation step, caseinates contain only trace amounts of lactose, making them somewhat unique amongst dairy ingredients.

Production and uses of whey proteins

Whey protein, which was traditionally treated as a waste by-product from cheesemaking, has been turned into a valuable product by concentrating the relative proportion of whey protein in the ingredient using membrane filtration or industrial chromatography techniques such as ion-exchange (Kulozik, 2009; Boland, 2011). Naming conventions for whey products depend on how much protein remains after the concentration process, with WPC or WPI referring to powders between 34-80% or >90% dry-basis protein respectively. Unfractionated WPC and WPI are now available as reasonably priced commodities, and have found wide-spread use in food applications due to their gelling, water-binding, and emulsification properties (Boland, 2011). WPI is also utilized extensively in sports nutrition products since it is quickly digested and relatively high in branched-chain-amino acids, which help stimulate protein synthesis in human muscles.

Recent innovations to whey fractionation technology have led to the isolation of very pure and valuable protein components from cheese whey. Examples include α-lactalbumin suitable for making high-quality infant formula, as well as glycomacropeptide that is a tolerable amino acid source for individuals afflicted with phenyl-ketonuria (Kulozik, 2009). These special fractionations are of increasing importance to dairy ingredient manufacturers as they attempt to add additional value to their whey products.

Production and uses of permeates

The term permeate refers to the sugars, organic and fatty acids, soluble minerals, and non-protein nitrogen compounds such as urea left after the production of high protein dairy powders (Skou et al., 2018). It is referred to as permeate since it contains all of the molecular species that are not retained (and thus pass through, or "permeate") the filtration membranes most commonly associated with dairy ingredient protein enrichment. Lactose composes the largest percentage of permeate, with dried whey permeate consisting of 65%-85% lactose (Smith et al., 2016). Permeate can be further categorized as whey or milk permeate depending on whether it was separated from whey or skim milk sources respectively (Smith et al., 2016). Permeates are often used for fertilizer, animal feed, standardization material for other dairy ingredients, and as a feedstock for the production of purified lactose (Hansen et al., 2010). Additionally, permeate makes a good fermentation feedstock for the production of lactic acid or ethanol (Smith et al., 2016). Permeates can also be used to reduce sodium in savory foods since compounds commonly found in permeate (e.g. potassium chloride and lactic, citric or orotic acid) are known to enhance salty flavors (Smith et al., 2016).

Production and uses of lactose

Purified lactose is available commercially in dried crystalline form, usually being refined from whey or whey permeate via crystallization (Wong and Hartel, 2014). Purified crystalline lactose is primarily used in pharmaceutical, infant nutrition, meat, and confectionary products (Paterson and Kellam, 2009). Pure lactose can also be used as a feedstock for many derivatives, including D-tagatose, galactooligosaccharides, lactitol, lactulose, and lactic acid (Paterson and Kellam, 2009).

Importance of lactose in dairy ingredients

Lactose presence can have a significant impact on the overall quality, value, and utility of dairy ingredients. The routine analysis of lactose in dairy ingredients could lead to improved utilization of dairy ingredients, as well as help to better understand lactose's role in ingredient quality. For example, measuring the lactose content of permeate and retentate streams can be a process optimization benchmark during membrane concentration of dairy proteins (Olabi, et al., 2015; Sluková et al., 2016). Also, incorporation of the correct amount of starting lactose is important for whey solids fermentation optimization (Sansonetti et al., 2010). Lastly, quality defects such as stickiness, caking, or excessive browning in dairy powders are largely dependent on the amount and form of lactose present in a dried ingredient (Olabi et al., 2015; Huppertz and Gazi, 2016; Gulzar and Jacquier, 2018).

Lactose Quantification Methods

Many tests exist to measure lactose in dairy products. Table 2-1 provides a non-exhaustive list of some documented lactose quantification methods and the objective shortcomings of each. Biosensors show the most promise for routine use due to their simplicity and swiftness. Since the BGM method proposed here is technically a biosensor-based method, the focus of this section will be to study biosensors from a general perspective in order to better understand how they work and what their shortcomings could be.

Table 2-1 Survey of lactose quantification methods for dairy foods

Reference(s)	Method identification	Objective shortcomings
Cheng and Christian, 1977	Redox Titration	Cumbersome, error prone.
Cheng and Christian, 1977	Phenol-sulphuric acid colorimetric assay	Non-specific detection & reaction chemistry.
Webb, 1974	Gravimetric precipitation of copper ions	Tedious sample preparation, interference from all reducing carbohydrates.
Idda et al., 2016	Gas chromatography (GC)	Expensive equipment, sample derivatization necessary, trained personnel needed
Gabbanini, et al., 2010; Upreti et al., 2006; AOAC 984.22	High-performance liquid chromatography (HPLC)	Expensive equipment, tedious sample preparation, trained personnel needed.
Lynch and Barbano, 2007	Calculation by difference	Mistakes other compounds for lactose, relies on the accuracy of multiple other tests.
Lynch and Barbano, 2007; AOAC 972.16	Infrared spectroscopy	Expensive equipment, non-specific, tedious & inflexible calibration required.
Lynch and Barbano, 2007; AOAC 2006.06	Enzymatic spectrophotometric absorbance	Expensive equipment, tedious sample preparation, trained personnel needed.
Silveira et al., 2016	Raman spectroscopy	Expensive equipment, nonspecific, tedious & inflexible calibration required.
Hu et al., 2007	Nuclear Magnetic Resonance (NMR)	Expensive equipment, trained personnel needed.
Jager et al., 2007	Electrophoresis	Expensive equipment, trained personnel needed.
Luzzana et al., 2001, 2003; Gille et al., 2018	Differential pH (Microlab EFA)	Requires diluted system to have a very specific buffering capacity.
Sharma and Leblanc, 2017	β-galactosidase based Biosensors	Non-commercial, difficulty automating immobilization technique.
Conzuelo et al., 2012; Glithero et al. , 2013; Lopez et al., 2017; Safina et al., 2010; Yakovleva et al., 2012	Cellobiose-Dehydrogenase based biosensors	Non-commercial, specificity issues with enzymes most commonly available.
Amamcharla and Metzger, 2011; Heinzerling et al., 2012	Blood glucose meter based biosensor	Requires specialized calibration, non-standardized equipment.
Churakova et al., 2019	BioMilk® ^{300} (a proprietary biosensor)	Workings are proprietary so not understood, works best for very low-lactose products.
-	LactoSens® (a proprietary biosensor)	Workings are proprietary so not understood.

Biosensors

Many conventional chemical (e.g. Spectroscopy, HPLC, etc.) and microbiological (e.g. plating) analyses used in the food industry are slow, expensive, require trained personnel, and often require tedious sample preparation procedures. Biosensors offer an attractive alternative to conventional measurements due to their specificity, simplicity, relatively low-cost, and possible portability (Mello and Kubota, 2002; Sharma and Leblanc, 2017).

Biosensors principally consist of a biological recognition element coupled to a transducer. In the presence of a target analyte, the biological recognition element creates a response and the transducer "translates" that response into an analytical signal that can be measured conveniently. Biologically produced signals and the transducer type that characterizes the name of common biosensors are summarized in table 2-2.

Table 2-2 Common biological signals and corresponding transducer types found in biosensors. Adapted from Mello & Kubota (2002).

Biologically produced signal	Typical transducer required
Electron tunneling, ion mobility, creation of a current, diffusion of electroactive or charged particles	Electrochemical (amperometric, potentiometric, etc.)
Temperature change	Thermal
Absorption or emission of electromagnetic radiation	Optical
Mass and/or microviscosity alterations of wave propagation	Piezoelectric

The biological recognition element in biosensors can be antibodies, enzymes, nucleic acids, micro-organisms, and living tissues. However, enzymes are used most often due to their speed and selectivity (Sharma and Leblanc, 2017). The biological recognition element is immobilized directly to a transducer most commonly operating under amperometry principles. Enzyme-based biosensors coupled with amperometry works as follows: First, the enzyme site facilitates the oxidation of a target analyte species, and an electrochemical current is subsequently produced. Then, this current is carried to an electrode (the transducer in this scheme) that has a constant potential applied to it, and the resulting current is proportional to analyte concentration (Mello and Kubota, 2002; Booth et al., 2017; Sharma and Leblanc, 2017).

How exactly the electrons from the oxidation reaction at the enzyme site reach the electrode dictates whether an amperometric biosensor is first, second or third generation as detailed in figure 2-6.

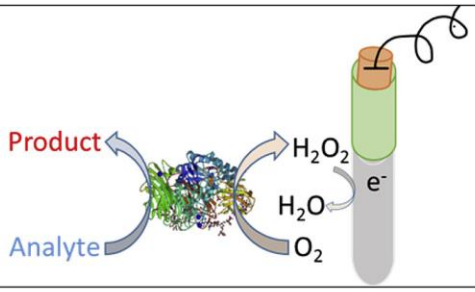
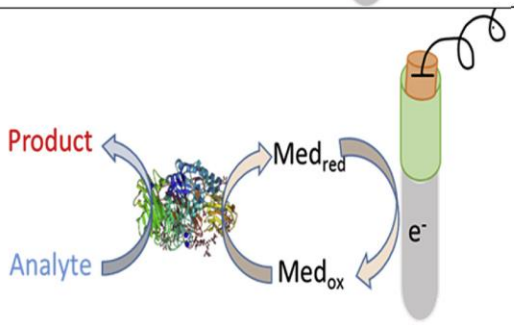
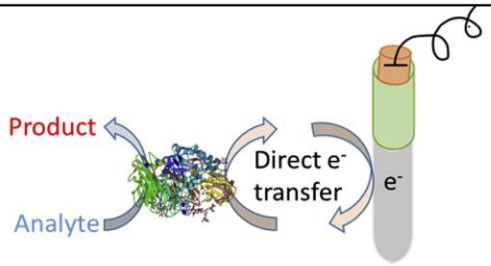
Generation	Schematic	Feature
First		The electrons are transferred to molecular oxygen and the resulting decrease in the oxygen concentration and/or the produced H_2O_2 is measured.
Second		Use artificial mediators or nanomaterials to transport electrons to the electrode.
Third		The electrons are transferred directly from enzyme to the electrode without any intermediate stages or use of mediators.

Figure 2-6 The three generations of enzyme based electrochemical amperometric biosensors. β-galactosidase is the biological recognition element in this example, but the principles are universal. Reprinted from Analytical Biochemistry, Vol. 535, Sharma. S.K & Leblanc, R.M. Biosensors based on β-galactosidase enzyme: Recent advances and perspectives, 1-11, Copyright (2017), with permission from Elsevier

Biosensors used in dairy foods

Biosensors are available for measuring many analytes of interest in milk, including hormones, antibiotics, pathogens, contaminants, and lactose. However, while there are many examples of these biosensors in the literature, there are disproportionately few commercial examples. This has been attributed to slow transfer of information from academia to industry, inability to reproducibly prepare biosensor surfaces, and matrix interferences of milk. The milk matrix itself seems to be the most major issue hindering the development of commercial biosensors for dairy products, as most commercially viable sensors would be based around electrochemical analyte quantification principles that are prone to interference from the many electroactive species in milk (Booth et al., 2017).

Biosensors used for lactose quantification

The most promising sensors for commercial lactose quantification have been based on enzymes in conjunction with amperometric transducers, reflecting general biosensor trends. Several examples of sensors with enzymatic recognition elements based around β- galactosidase in conjunction with other enzymes have been used to quantify lactose in milk, blood, and wastewater. However, as of 2017, it was found to be difficult to commercially immobilize the enzyme cocktails in these types of sensors to the transducer (Sharma and Leblanc, 2017). Another promising group of sensors are those based around a cellobiose dehydrogenase (CDH) enzymatic recognition element, which have successfully been applied to measuring lactose in milk, yogurt, and cheese plant-waste water streams (Safina et al., 2010; Yakovleva et al., 2012; Lopez et al., 2017; Glithero et al., 2013). However, the CDH enzyme was not commercially available as late as 2012 (Conzuelo et al., 2012), and CDH with the greatest commercial viability

have shown some activity towards glucose and other sugars when complex sensor manufacturing strategies are not employed (Lopez et al., 2017).

Despite setbacks reported in the literature, there are some proprietary commercial biosensors available to quantify lactose, including the Lactosens® (DirectSens GMBH, marketed by Chr. Hansen in the United States), BioMilk® ^{300} (Biolan, marketed by DSM), and YSI biochemistry analyzer (Xylem inc.). Both the BioMilk® ^{300} and Lactosens® are advertised as being 3rd party validated against a reference method, and the BioMilk® ^{300} was recently documented as being an excellent tool for measuring low (<0.3%) lactose levels in lactose-free milks (Churakova et al., 2019). Also, the author had the opportunity to evaluate a Lactosens® unit and found the unit to be accurate relative to an HPLC reference method for lactose measurement in dried dairy ingredient samples when properly equipped (personnel communication).

Proprietary options aside, blood glucose meter (BGM) biosensors typically associated with at-home diabetes care have been adapted for measuring lactose in milk (Amamcharla and Metzger, 2011) and in model lactose solutions (Heinzerling et al., 2012). The primary advantage of a BGM method over the aforementioned proprietary options would be lower costs, with meters typically demanding a one-time investment of ~$20 USD and individual test strips often costing <$0.50 USD each.

Blood glucose meter biosensors

Blood glucose meter (BGM) biosensors are very adaptable to uses outside of their original design, having been shown to quantify not only lactose but also enzymes like β-galactosidase or pathogens such as influenza virus and *E. coli* when appropriate sample preparation is performed (Das et al., 2018). Dairy product manufactures could readily adopt the

BGM for routine lactose monitoring activities given their commercial availability, simple use, small size, and very low cost. However, an understanding of BGM operating principles and theory is necessary to select an appropriate BGM for lactose analysis. A better understanding of how BGM biosensors work will also help clarify what sample matrix characteristics are important to account for in a generalized method for lactose analysis in dairy products beyond milk.

Blood glucose meter operating principles

BGM biosensors are key tools for patient and health care provider management of diabetes mellitus (Lan et al., 2011; Link et al., 2015; Sode et al., 2017). They are available over the counter at most drugstores, are fast and simple to operate, and require very small sample sizes (Vanavanan et al., 2010). Proper operation of the meters takes little training, with an operator needing only to insert a new test strip into the corresponding BGM, and then dripping sample onto the capillary site of the test strip; glucose quantification results are usually achieved in < 5 seconds.

Most commercial BGM biosensors use an enzymatic biological recognition element. Common enzymes used include glucose oxidase (GOx) or glucose dehydrogenase (GDH) coupled with nicotinamide adenine dinucleotide or flavin adenine dinucleotide (referred to as GDH-NAD or GDH-FAD respectively) as co-factors (Sode et al., 2017). Most commercial meters available are 1^{st} or 2^{nd} generation amperometric biosensors in principle; glucose is selectively oxidized by the enzyme and mediators transfer the created electrochemical current to an electrode that is under an applied voltage. The resulting current is proportional to glucose concentration. An example of this scheme in a GOx-based amperometric biosensor is shown in

figure 2-7.

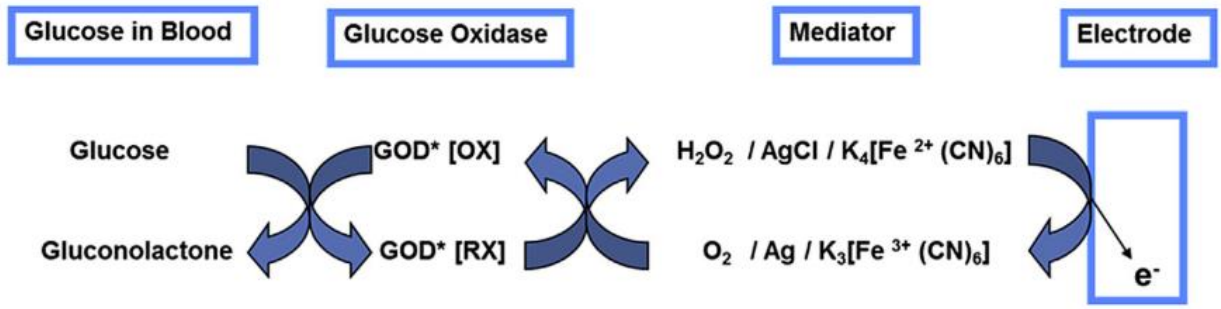


Figure 2-7 Glucose measurement scheme of a typical amperometric glucose oxidase-based blood glucose meter. Figure reproduced as-is from Wang and Lee (2015) under permission of the creative commons license CC BY-NC-ND 4.0

Potential issues with a blood glucose meter-based lactose assay

One key issue associated with a BGM based lactose assay is the lack of standardized tools. There is no certification or assurance that a BGM will perform adequately in any application outside its intended use for blood. Instead, validation of results falls upon the user and associated third parties if desired. A certain level of manufacturing quality for commercial BGM sensors is assured via compliance to International Organization for Standardization (ISO) standard ISO 15197 that is updated periodically, most recently ISO 15197-2013 (Pfützner et al., 2012; Klonoff, 2014; Link et al., 2015). However, these compliances are largely meaningless outside of a meter's intended use, so more attention should be paid to the actual design and operation principles of the BGM to choose the correct tools.

When selecting a BGM for use in a lactose assay, selection of the correct meter begins with assessing what technologies are available and what can act as an interferent. According to manufacture instructions and Link et al. (2015), most BGM brands are rated for measuring glucose concentrations between 20- 600 mg/ dL, temperatures between 10 – 40 C°, relative humidity between 10-85 %, and hematocrit levels between 10-60 %. These parameters are

relevant for blood, but they offer guidance when establishing quantification procedures for other matrices like dairy ingredients. The next step is to consider known interferences since BGM performance can be impacted by a variety of environmental conditions and non-glucose compounds. Table 2-3 lists common enzyme recognition elements used in BGM biosensors and known measurement interferents for each of those technologies.

Table 2-3 Measurement environment and non-glucose compound interferents known to bias blood-glucose meter readings.

Reference(s)	Enzyme recognition element	Measurement environment interferents	Non-glucose compound interferents
Dungan et al., 2007;Ramljak et al., 2013); de Mol et al., 2010)	Glucose oxidase (GOx)	Hematocrit, pH, temperature, humidity, atmospheric oxygen levels	Ascorbic acid, acetaminophen, mannitol
Dungan et al., 2007;Ramljak et al., 2013	Glucose dehydrogenase-Flavin adenine dinucleotide (GDH-FAD)	Hematocrit, pH, temperature, humidity	Ascorbic acid, acetaminophen, dopamine
Dungan et al., 2007;Perera et al., 2011; Ramljak et al., 2013	Glucose dehydrogenase-Nicontinamide adenine dinucleotide (GDH-NAD)	Hematocrit, pH, temperature, humidity	Ascorbic acid, acetaminophen, dopamine, maltose, maltotriose, maltotetrose, xylose
Dungan et al., 2007; Schleis, 2007; Perera et al., 2011; Ramljak et al., 2013	Glucose dehydrogenase-Pyrroloquinoline quinone (GDH-PQQ)	Hematocrit, pH, temperature, humidity	Ascorbic acid, acetaminophen, dopamine, maltose, maltotriose, maltotetrose, galactose, xylose

Universal interferents, whether they be environmental or compound specific, must be considered regardless of what BGM is used. Universal environmental interferents such as pH, temperature, humidity, and hematocrit all have an impact on the enzymatic recognition element:

Glucose measurements in blood outside the pH range of 6.8-7.8 are inaccurate due to changes in the sensing enzyme's activity (Dungan et al., 2007). Temperature, and to a lesser extent humidity, impact enzymatic activity (Erbach et al., 2016). High hematocrit levels (volume of red blood cells relative to plasma volume in blood) has been shown to artificially lower the BGM output for glucose measurements in blood due to physical hinderance of the test strip's enzyme site, and some BGM brands employ algorithms to correct for the effect (Ramljak et al., 2013).

Compounds that universally interfere with amperometric BGM measurements include ascorbic acid and acetaminophen due to the ease at which these compounds are non-specifically oxidized by the BGM electrode's reference voltage (Lan et al., 2011). While these compounds are usually not of concern in dairy ingredients, the principle that any easily oxidized substance can bias BGM output must be respected.

Interferents can also be specific to a given BGM technology. For example, GOx-based sensor output can be artificially lowered in areas where oxygen is sparse (i.e. high altitude locations) due to these meters getting their molecular oxygen mediator from the environment (de Mol et al., 2010). Also, galactose and certain small glucose polymers are interfering compounds for glucose dehydrogenase- nicontinamide adenine dinucleotide (GDH-NAD) and glucose dehydrogenase- pyrroloquinoline quinone (GDH-PQQ) based BGM biosensors. In general, attention must be paid to the BGM technology being used in order to select a BGM that will be optimal for the intended application.

Conclusions

Lactose is a sugar with unique functional properties and is of great consequence to the nutritional value and marketability of dairy products. Lactose is a ubiquitous component of dairy ingredients and plays a principle role in the quality and shelf life of dried dairy ingredients

containing great amounts of the substance. Many processes involving dairy ingredient utilization and manufacture could benefit from routine application of an accurate, rapid, and low-cost way to quantify lactose. BGM biosensors have been identified as a potential tool to fit this need. However, there are several key items that need to be investigated to create a generalized BGM-based lactose assay for dairy ingredients.

Firstly, since BGM-sensors can only quantify a certain range of glucose concentrations, different dilution schemes tailored to accommodate the wide range of lactose contents available in ingredients will be necessary. This dictates that relevant measurement environment factors such as pH or non-lactose solids amount and composition must be studied to create a robust lactose assay for dairy ingredients.

Secondly, great care must be put into selecting BGM brands that are appropriate for measuring lactose. For example, GDH-PQQ-based meters are inappropriate since galactose is an interfering analyte for these meters, and galactose will be generated during the mandatory lactose hydrolysis step of the proposed BGM analysis. Also, some meters are known to algorithmically correct for different hematocrit levels, and this may be useful for lactose analysis in dairy ingredients assuming that non-lactose solids such as protein would have an analogous effect as red blood cells on BGM output. Investigation of several BGM-brands with differences in their underlying technology may reveal a meter that is optimal for a BGM-based lactose analysis specific for dairy ingredients.

References

Agarwal, S., R.L.W. Beausire, S. Patel, and H. Patel. 2015. Innovative uses of milk protein concentrates in product development. J. Food Sci. 80:A23–A29. doi:10.1111/1750-3841.12807.

Amamcharla, J.K., and L.E. Metzger. 2011. Development of a rapid method for the measurement of lactose in milk using a blood glucose biosensor. J. Dairy Sci. 94:4800–4809. doi:10.3168/jds.2011-4416.

AOAC. 2012. Official Methods of Analysis. 19th ed. AOAC, Gaitherburg, MD.

Boland, M. 2011. Whey proteins. G.O. Phillips and P.A. Williams, ed. Woodhead Publishing, Philadelphia.

Booth, M.A., H. Karaosmanoglu, Y. Wu, and A. Partridge. 2017. Biosensor platforms for detecting target species in milk samples. M.U. Ahmed, M. Zourob, and E. Tamiya, ed. Royal Society of chemistry, Cambridge.

Cheng, F.S., and G.D. Christian. 1977. Rapid Enzymatic Determination of Lactose in Food Products Using Amperometric Measurement of the Rate of Depletion of Oxygen. Analyst 102:124–131.

Churakova, E., K. Peri, J.S. Vis, D.W. Smith, J.M. Beam, M.P. Vijverberg, M.C. Stor, and R.T. Winter. 2019. Accurate analysis of residual lactose in low-lactose milk: comparing a variety of analytical techniques. Int. Dairy J.. doi:10.1016/j.idairyj.2019.02.020.

Conzuelo, F., A.J.A. Reviejo, and J.M.J. Pingarron. 2012. Lactose in milk and dairy products : A focus on biosensors. V. Preedy, ed. Royal Society of chemistry, Cambridge, U.K.

Corradini, C., A. Cavazza, and C. Bignardi. 2012. High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection as a Powerful Tool to Evaluate Carbohydrates of Food Interest: Principles and Applications. Int. J. Carbohydr. Chem. 2012:1–13. doi:10.1155/2012/487564.

Das, A., X. Cui, V. Chivukula, and S.S. Iyer. 2018. Detection of Enzymes, Viruses, and Bacteria Using Glucose Meters. Anal. Chem. 90:11589–11598. doi:10.1021/acs.analchem.8b02960.

Dirienzo, D. 2016. Research gaps in the use of dairy ingredients in food aid products. Food Nutr. Bull. 37:S51–S57. doi:10.1177/0379572116628351.

Dungan, K., J. Chapman, S.S. Braithwaite, and J. Buse. 2007. Glucose measurement: Confounding issues in setting targets for inpatient management. Diabetes Care 30:403–409. doi:10.2337/dc06-1679.

Durham, R.J.J. 2009. Modern approaches to lactose production. 1st ed. M. Corredig, ed.

Woodhead Publishing, Boca Raton, FL

Erbach, M., G. Freckmann, R. Hinzmann, B. Kulzer, R. Ziegler, L. Heinemann, and O. Schnell. 2016. Interferences and limitations in blood glucose self-testing: An overview of the current knowledge. J. Diabetes Sci. Technol. 10:1161–1168. doi:10.1177/1932296816641433.

Gabbanini, S., E. Lucchi, F. Guidugli, R. Matera, and L. Valgimigli. 2010. Anomeric discrimination and rapid analysis of underivatized lactose, maltose, and sucrose in vegetable matrices by U-HPLC - ESI-MS/MS using porous graphitic carbon. J. Mass Spectrom. 45:1012–1018. doi:10.1002/jms.1750.

Gille, D., B. Walther, R. Badertscher, A. Bosshart, C. Brügger, M. Brühlhart, R. Gauch, P. Noth, G. Vergères, and L. Egger. 2018. Detection of lactose in products with low lactose content. Int. Dairy J.. doi:10.1016/j.idairyj.2018.03.003.

Glithero, N., C. Clark, L. Gorton, W. Schuhmann, and N. Pasco. 2013. At-line measurement of lactose in dairy-processing plants. Anal. Bioanal. Chem. 405:3791–3799. doi:10.1007/s00216-012-6598-y.

Gulzar, M., and J.C. Jacquier. 2018. Impact of Residual Lactose on Dry Heat-Induced Pre-texturization of Whey Proteins. Food Bioprocess Technol. 11:1985–1994. doi:10.1007/s11947-018-2162-x.

Hansen, C.L., F. van den Berg, M.A. Rasmussen, S.B. Engelsen, and S. Holroyd. 2010. Detecting variation in ultrafiltrated milk permeates - Infrared spectroscopy signatures and external factor orthogonalization. Chemom. Intell. Lab. Syst. 104:243–248. doi:10.1016/j.chemolab.2010.08.014.

Heinzerling, P., F. Schrader, and S. Schanze. 2012. Measurement of enzyme kinetics by use of a blood glucometer: Hydrolysis of sucrose and lactose. J. Chem. Educ. 89:1582–1586. doi:10.1021/ed200735f.

Horner, T.W., M.L. Dunn, D.L. Eggett, and L.V. Ogden. 2011. β-Galactosidase activity of commercial lactase samples in raw and pasteurized milk at refrigerated temperatures. J. Dairy Sci. 94:3242–3249. doi:10.3168/jds.2010-3742.

Hu, F., K. Furihata, Y. Kato, and M. Tanokura. 2007. Nondestructive quantification of organic compounds in whole milk without pretreatment by two-dimensional NMR spectroscopy. J. Agric. Food Chem. 55:4307–4311. doi:10.1021/jf062803x.

Huber, K.C., and J.N. BeMiller. 2017. Carbohyrdates. 5th ed. D. Srinivasan and K.L. Parkin, ed. CRC Press, Baton Rouge.

Huppertz, T., and I. Gazi. 2016. Lactose in dairy ingredients: Effect on processing and storage stability. J. Dairy Sci. 99:6842–6851. doi:10.3168/jds.2015-10033.

Idda, I., N. Spano, M. Ciulu, V.M. Nurchi, A. Panzanelli, M.I. Pilo, and G. Sanna. 2016. Gas chromatography analysis of major free mono- and disaccharides in milk: Method assessment, validation, and application to real samples. J. Sep. Sci. 39:4577–4584. doi:10.1002/jssc.201600583.

Jager, A. V., F.G. Tonin, and M.F.M. Tavares. 2007. Comparative evaluation of extraction procedures and method validation for determination of carbohydrates in cereals and dairy products by capillary electrophoresis. J. Sep. Sci. 30:586–594. doi:10.1002/jssc.200600370.

Klonoff, D.C. 2014. Point-of-care blood glucose meter accuracy in the hospital setting. Diabetes Spectr. 27:174–179. doi:10.2337/diaspect.27.3.174.

Kulozik, U. 2009. Novel approaches for the separation of dairy components and manufacture of dairy ingredients. M. Corredig, ed. Woodhead Publishing, Boca Raton.

Lan, K., K. McAferty, P. Shah, E. Lieberman, D.R. Patel, C.B. Cook, and J.T. La Belle. 2011. A disposable tear glucose biosensor - Part 3: Assessment of enzymatic specificity. J. Diabetes Sci. Technol. 5:1108–1115. doi:10.1177/193229681100500511.

Link, M., C. Schmid, S. Pleus, A. Baumstark, D. Rittmeyer, C. Haug, and G. Freckmann. 2015. System accuracy evaluation of four systems for self-monitoring of blood glucose following ISO 15197 using a glucose oxidase and a hexokinase-based comparison method. J. Diabetes Sci. Technol. 9:1041–1050. doi:10.1177/1932296815580161.

Lopez, F., S. Ma, R. Ludwig, W. Schuhmann, and A. Ruff. 2017. A polymer multilayer based amperometric biosensor for the detection of lactose in the presence of high concentrations of glucose. Electroanalysis 29:154–161. doi:10.1002/elan.201600575.

Luzzana, M., D. Agnellini, P. Cremonesi, and G. Caramenti. 2001. Enzymatic reactions for the determination of sugars in food samples using the differential pH technique. Analyst 126:2149–2152. doi:10.1039/b106880f.

Luzzana, M., D. Agnellini, P. Cremonesi, G. Caramenti, and S. De vita. 2003. Milk lactose and lactulose determination by the differential pH technique. Le Lait, INRA Ed. 83:409–416. doi:10.1051/lait.

Lynch, J., and D.M. Barbano. 2007. Determination of the lactose content of fluid milk by spectrophotometric enzymatic analysis using weight additions and path length adjustment: Collaborative study. J. Aoac Int. 90:196–216.

McCarthy, K.S., M. Parker, A. Ameerally, S.L. Drake, and M.A. Drake. 2017. Drivers of choice for fluid milk versus plant-based alternatives: What are consumer perceptions of fluid milk?. J. Dairy Sci. 100:6125–6138. doi:10.3168/jds.2016-12519.

McSweeney, P.L.H., and P.F. Fox. 2009. Advanced Dairy Chemistry Volume 3: Lactose, Water, Salts and Minor Constituents. 3rd ed. Springer, New York.

Mello, L.D., and L.T. Kubota. 2002. Review of the use of biosensors as analytical tools in the food and drink industries. Food Chem. 77:237–256. doi:10.1016/S0308-8146(02)00104-8.

de Mol, P., H.G. Krabbe, S.T. de Vries, M.J. Fokkert, B.D. Dikkeschei, R. Rienks, K.M. Bilo, and H.J.G. Bilo. 2010. Accuracy of handheld blood glucose meters at high altitude. PLoS One 5:1–7. doi:10.1371/journal.pone.0015485.

O'Kennedy, B. 2011. Caseins. G.O. Phillips and P.A. Williams, ed. Woodhead Publishing, Philadelphia, PA.

Olabi, A., S. Jinjarak, R. Jiménez-Flores, J.H. Walker, and H. Daroub. 2015. Compositional and sensory differences of products of sweet-cream and whey buttermilk produced by microfiltration, diafiltration, and supercritical CO2. J. Dairy Sci. 98:3590–3598. doi:10.3168/jds.2014-9141.

Paterson, A.H.J.H.J., and S.J.J. Kellam. 2009. Transformation of lactose for value-added ingredients. M. Corredig, ed. Woodhead Publishing, Boca Raton, FL.

Perera, N.J., P.M. Stewart, P.F. Williams, E.L. Chua, D.K. Yue, and S.M. Twigg. 2011. The danger of using inappropriate point-of-care glucose meters in patients on icodextrin dialysis. Diabet. Med. 28:1272–1276. doi:10.1111/j.1464-5491.2011.03362.x.

Pfützner, A., M. Mitri, P.B. Musholt, D. Sachsenheimer, M. Borchert, A. Yap, and T. Forst. 2012. Clinical assessment of the accuracy of blood glucose measurement devices. Curr. Med. Res. Opin. 28:525–531. doi:10.1185/03007995.2012.673479.

Ramljak, S., J.P. Lock, C. Schipper, P.B. Musholt, T. Forst, M. Lyon, and A. Pfützner. 2013. Hematocrit interference of blood glucose meters for patient self-measurement.. J. Diabetes Sci. Technol. 7:179–89. doi:10.1177/193229681300700123.

Safina, G., R. Ludwig, and L. Gorton. 2010. A simple and sensitive method for lactose detection based on direct electron transfer between immobilised cellobiose dehydrogenase and screen-printed carbon electrodes. Electrochim. Acta 55:7690–7695. doi:10.1016/j.electacta.2009.10.052.

Sansonetti, S., S. Curcio, V. Calabrò, and G. Iorio. 2010. Optimization of ricotta cheese whey (RCW) fermentation by response surface methodology. Bioresour. Technol. 101:9156–9162. doi:10.1016/j.biortech.2010.07.030.

Schleis, T.G. 2007. Interference of maltose, icodextrin, galactose, or xylose with some blood glucose monitoring systems.. Pharmacotherapy 27:1313–1321. doi:10.1592/phco.27.9.1313.

Schuck, P. 2009. Understanding the factors affecting spray-dried dairy powder properties and behavior. M. Corredig, ed. Woodhead Publishing, Boca Raton, FL.

Sharma, S.K., and R.M. Leblanc. 2017. Biosensors based on β-galactosidase enzyme: Recent advances and perspectives. Anal. Biochem. 535:1–11. doi:10.1016/j.ab.2017.07.019.

Silveira, L., E.D.C.M. Motta, R.A. Zângaro, M.T.T. Pacheco, C.J. De Lima, and L.H. Moreira. 2016. Characterization of nutritional parameters in bovine milk by Raman spectroscopy with least squares modeling. Instrum. Sci. Technol. 44:85–97. doi:10.1080/10739149.2015.1055578.

Skou, P.B., B. Khakimov, T.H. Hansen, S.D. Aunsbjerg, S. Knøchel, D. Thaysen, and F. van den Berg. 2018. Chemical characterization by gas chromatography-mass spectrometry and inductively coupled plasma-optical emission spectroscopy of membrane permeates from an industrial dairy ingredient production used as process water. J. Dairy Sci. 101:135–146. doi:10.3168/jds.2017-12950.

Sluková, M., A. Hinková, S. Henke, F. Smrž, M. Lukačíková, V. Pour, and Z. Bubník. 2016. Cheese whey treated by membrane separation as a valuable ingredient for barley sourdough preparation. J. Food Eng. 172:38–47. doi:10.1016/j.jfoodeng.2015.03.023.

Smith, S.T., L. Metzger, and M.A. Drake. 2016. Evaluation of whey, milk, and delactosed permeates as salt substitutes. J. Dairy Sci. 99:8687–8698. doi:10.3168/jds.2016-10904.

Sode, K., N. Loew, Y. Ohnishi, H. Tsuruta, K. Mori, K. Kojima, W. Tsugawa, J.T. LaBelle, and D.C. Klonoff. 2017. Novel fungal FAD glucose dehydrogenase derived from Aspergillus niger for glucose enzyme sensor strips. Biosens. Bioelectron. 87:305–311. doi:10.1016/j.bios.2016.08.053.

Upreti, P., L.L. McKay, and L.E. Metzger. 2006. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Changes in residual sugars and water-soluble organic acids during ripening. J. Dairy Sci. 89:429–443. doi:10.3168/jds.S0022-0302(06)72107-5.

Vanavanan, S., P. Santanirand, U. Chaichanajarernkul, A. Chittamma, J.A. DuBois, T. Shirey, and M. Heinz. 2010. Performance of a new interference-resistant glucose meter. Clin. Biochem. 43:186–192. doi:10.1016/j.clinbiochem.2009.09.010.

Wang, H.C., and A.R. Lee. 2015. Recent developments in blood glucose sensors. J. Food Drug Anal. 23:191–200. doi:10.1016/j.jfda.2014.12.001.

Webb, B.H. 1974. Fundamentals of Dairy Chemistry. 2d edition. A.H. Johnson and J.A. Alford, ed. Avi Pub. Co., Westport, CT.

Wong, S.Y., and R.W. Hartel. 2014. Crystallization in Lactose Refining-A Review. J. Food Sci. 79:257–272. doi:10.1111/1750-3841.12349.

Yakovleva, M., O. Buzas, H. Matsumura, M. Samejima, K. Igarashi, P.O. Larsson, L. Gorton, and B. Danielsson. 2012. A novel combined thermometric and amperometric biosensor for lactose determination based on immobilised cellobiose dehydrogenase. Biosens. Bioelectron. 31:251–256. doi:10.1016/j.bios.2011.10.027.

Chapter 3 - Research objectives

This study focused on developing a rapid, low-cost lactose analysis for dairy ingredients utilizing blood glucose meter (BGM) biosensors. The study was conducted in 4 phases, each one investigating what considerations are important for providing robust lactose measurements in dairy ingredient systems, as well as holistically indicating a particular BGM brand that may be optimal for these measurements. The specific objectives, in phase order, are as follows:

1. Explore how solution pH and total lactose concentration may affect BGM output bias.
2. Investigate the effect that different types and amounts of non-lactose solids may have on BGM output bias.
3. Create model ingredient-specific calibration curves that holistically represent one possible dilution scheme tailored for lactose quantification in common dairy ingredients; subsequently validate the accuracy of these standard curves with an independently prepared series of model dairy ingredient dilutions.
4. Test the robustness of the developed method using a sampling of commercial dairy ingredients.

Chapter 4 - Adapting blood glucose meter biosensors to the measurement of lactose in dairy ingredients*

Abstract

Commonly used lactose assays (enzymatic spectrophotometric absorbance [EZA] and HPLC) for dairy ingredients are relatively expensive and time-consuming. A blood glucose meter (BGM)-based method has been successfully documented as a rapid lactose assay in milk. The BGM method involves diluting a sample to an appropriate level with buffer, adding lactase enzyme, incubating at 40°C for ~15 minutes, and measuring the resulting glucose content with the BGM. A standard curve developed between the known lactose concentration and BGM reading is used to quantify lactose in unknown samples. The objective of this study was to evaluate the BGM-based lactose analysis method in whey- and skim milk-derived (WD and SMD, respectively) ingredients and was done in 4 phases. In phase 1, the effect of pH and lactose concentration on the BGM reading was investigated using a factorial design with 2 factors (pH: 6.05-6.94 and lactose: 0.2% or 0.4%) and found that BGM readings are significantly ($P<0.05$) affected by lower pH values at both lactose levels. In phase 2, the effect of total solids and ingredient type was investigated using a factorial design with 2 factors (ingredient type: WD or SMD and total solids: 0-8%). It was observed that the BGM reading was significantly ($P<0.05$) affected by ingredient type and total solids. Phase 3 involved developing a linear relationship between the BGM reading and an EZA reference method to ascertain the accuracy of the proposed BGM method. Different ingredient type (WD or SMD) and non-lactose solids (0.5-27%) model ingredient dilutions prepared over a range of lactose contents (0.08-0.62%) were measured using the BGM and EZA methods. The average absolute percent bias difference between the BGM method and EZA reference method results was found to be between 2.2-7.3%

depending on what meter, test strip lot, and dilution model was being measured. In phase 4, 15 samples procured from commercial sources ranging from 0.01-81.9% lactose were evaluated using the BGM method and EZA reference method. Average absolute percent bias differences in lactose results between the two method were between 3.6-5.0% and 5.3-9.7% for a well- and poorly - performing meter respectively. Overall, the BGM method is a promising tool for rapid, low-cost analysis of lactose in both high-lactose and low-lactose dairy ingredients.

** To be submitted to the Journal of Dairy Science*

Introduction

Lactose, a disaccharide sugar composed of glucose and galactose moieties linked by a β-1,4 glyosidic bond, is the principle carbohydrate present in dairy products (Huber and BeMiller, 2017). Lactose is a nutritionally important part of many dairy foods, being of upmost importance in infant formula (Paterson and Kellam, 2009) as well as conferring valuable calcium absorption and enteric prebiotic growth benefits to the dairy consumer (Dirienzo, 2016). Conversely, the majority of the world's human population loses the ability properly digest lactose upon reaching adult hood, so the development and sale of more lactose-free products could be a large growth opportunity for the dairy industry (McCarthy et al., 2017).

Lactose is a ubiquitous constituent of most dried dairy ingredients, comprising <2% of dry protein isolates to 100% of pure lactose powders (Huppertz and Gazi, 2016). Common dairy ingredients come in two broad categories, being derived from either whey or skim milk. The proportion of lactose in an ingredient is usually inversely related to the protein content of an ingredient, with the primary goal of protein isolate and concentrate manufacture being to remove lactose, fat and minerals. The routine analysis of lactose in dairy ingredients could lead to improved utilization of dairy ingredients, as well as help to better understand lactose's role in ingredient quality. For example, measuring the lactose content of permeate and retentate streams can be a valuable troubleshooting tool during the membrane concentration of dairy proteins (Olabi, et al., 2015; Sluková et al., 2016). Likewise, incorporation of the correct amount of starting lactose is important for whey solids fermentation optimization (Sansonetti et al., 2010). Lastly, quality defects such as stickiness, caking, or excessive browning in dairy powders are largely dependent on the amount and form of lactose present in a dried ingredient (Olabi et al., 2015; Huppertz and Gazi, 2016; Gulzar and Jacquier, 2018).

Current common methods of lactose analysis include mid-infrared spectroscopy for holistic composition testing in milk (Margolies and Barbano, 2017) in addition to HPLC (Gabbanini, et al., 2010; Upreti et al., 2006) and enzymatic spectrophotometric absorbance assays (Lynch and Barbano, 2007) that are useful for more specific lactose testing in most dairy foods. These established methods of lactose analysis have shortcomings such as tedious sample preparation or expensive equipment requirements that can be alleviated using biosensors, which are generally accepted as being faster, easier, and lower cost to use (Conzuelo et al., 2012; Glithero et al., 2013; Sharma and Leblanc, 2017). However, there are few commercial biosensors available specifically for lactose analysis in dairy foods due largely to the complexity of milk as a measurement matrix, as well as difficulties associated with commercializing promising technologies (Booth et al., 2017). Only recently has a commercial biosensor designed for lactose been documented in the literature, the Biomilk® 300, which is tailored for the analysis of residual lactose in lactose free-dairy products (Churakova et al., 2019). Another commercial biosensor, the Lactosens®, was recently evaluated by the author and found to accurately measure lactose in some dried dairy ingredients when properly equipped (personal communication).

As an alternative to more expensive commercial lactose biosensors, blood glucose meter (BGM) biosensors commonly associated with glucose monitoring in diabetic patients can be adapted to the measurement of lactose (Amamcharla and Metzger, 2011; Heinzerling, et al., 2012). BGM biosensors are easy to use, low cost, and widely available (Vanavanan et al., 2010). Any BGM is a possible candidate for measuring lactose in dairy ingredients so long as the enzymatic detection element used is glucose oxidase, glucose dehydrogenase nicotinamide adenine dinucleotide (GDH-NAD), or glucose dehydrogenase flavin adenine dinucleotide (GDH-FAD). Glucose dehydrogenase pyrroloquinolinequinone (GDH-PQQ) or glucose dye

oxidoreductase based BGM sensors should not be used since galactose is a known interferent for these meters (Frias et al., 2010; Schleis, 2007), which is problematic given that the proposed method relies on the hydrolysis of lactose into glucose and galactose. Additionally, some sample matrix and environment-dependent interferents known to bias glucose measurements in blood may extrapolate to lactose measurements in dairy ingredients and thus must be accounted for in the proposed analysis method: A sample pH< 6.8 is known to erroneously lower BGM output for glucose measurements in blood (Dungan et al., 2007). Varying hematocrit levels (volume of red blood cells relative to plasma volume in blood) have been shown to bias the BGM output for glucose measurements in blood on a hematocrit-proportional basis due to physical hinderance of the test strip's active enzyme site (Ramljak et al., 2013). BGM measurement accuracy can be affected by environmental temperature and, to a lesser extent, humidity (Erbach et al., 2016).

To create a robust BGM-based lactose analysis, strategies to compensate for potential sample matrix dependent interferents such as pH and hematocrit (the dairy ingredient analogy being different amounts of non-lactose solids present in diluted samples) must be explored in this work. Also, it is desirable to discover an optimal BGM tool to use given the variety of possible tools available in the marketplace.

Materials and methods

Experimental Design

The efficacy of the proposed blood glucose meter (BGM) method was shown in 4 phases. In phase 1, the effect of pH and lactose concentration on BGM output bias was investigated. In phase 2, BGM measurements were performed on different dairy ingredient type and total solids combinations to study the effect these combinations could have on BGM output bias. The findings of phases 1 and 2 were applied to phase 3, where a calibration procedure for lactose

quantification in model dairy ingredient dilutions was verified by analyzing the linear relationship of paired lactose results between the proposed BGM method and a reference method. The developed method was then used to quantify the lactose content of commercial dairy ingredients in phase 4 to assess the robustness of the proposed method.

Buffers, dairy ingredients, and solution preparation

All solutions were made using 1X phosphate buffered saline (PBS) buffer prepared by diluting 10X PBS stock (Fischer Scientific, Pittsburgh, PA) with deionized (DI) water. Hydrochloric acid of 1N concentration (Acros Organics, Pittsburgh, PA) was used to titrate all buffers to their target pH (pH=7 unless otherwise indicated). A calibrated accumet® XL150 pH meter (Fischer Scientific, Pittsburgh, PA) was used for all pH measurements. ACS-certified grade α-lactose monohydrate (Fischer Scientific, Pittsburgh, PA) was used to adjust the lactose content of any model dilutions prepared. Whey- or skim milk-derived (WD or SMD respectively) ingredients described in this work were a gift from Agropur Ingredients (La Crosse, WI), and were obtained in dry form. All powders were mixed into ~20 °C buffer using an RPM-controlled mixer equipped with a propeller type spindle for 10 minutes, and the resulting dispersions were aged between 1 - 4 °C for at least 24 hours before any analysis work was performed.

Lactase enzyme

Enzeco ® lactase NL β-galactosidase, or lactase, enzyme used in this work was obtained from the Enzyme Development Corporation (New York, New York). According to the material specification data, the enzyme was prepared from a dairy yeast belonging to the *kluyveromyces* genus, had an activity level of 2450-3150 yeast lactase units/g, and achieves maximum lactose hydrolysis rates at incubation temperatures between 31-40 °C and pH between 6-7.

Blood glucose meter measurements

The BGM units used in this work include the ReliOn[†] prime (Wal-Mart stores, Bentonville AR), Nova Max Plus[††] (Nova Biomedical Corp, Waltham MA), OneTouch Verio Flex[†††] (LifeScan Europe, Zug Switzerland), and the FreeStyle Precision Neo[††††] (Abbott Diabetes Care Ltd., Witney UK). All units were capable of measuring at least 20- 500 mg/dL (0.02-0.5%) glucose in blood. To ensure proper functioning, new test strip lots were tested using the manufacture recommended control glucose solutions before any further measurements took place.

No preparation of samples beyond dilution is required for BGM lactose analysis. The BGM lactose analysis proposed here involved weighing out 5.0g of diluted sample into a test tube, thoroughly mixing the sample with 0.1g (~245 yeast lactase units) lactase enzyme, incubating the mixed sample at 40 °C for ≥15 mins, and then measuring the created glucose content by pipetting the incubated sample directly onto the capillary site of a BGM test strip. To maintain a consistent sample temperature, dilutions were removed from the incubation water bath immediately before measurement, and then promptly replaced into the water bath. During BGM measurements performed in this work, the room temperature was controlled to between 22-24 °C, and ambient humidity measured between <20% - 39%.

[†]"ReliOn" is a trademark of Wal-mart stores, Bentonville AR

[††]"Nova Max" is a registered trademark of Nova Biocmedical Corp, Waltham, MA. "Plus" is a trademark of the Nova Biomedical Corp.

[†††]"OneTouch" is a registered trademark of LifeScan Europe, Swizerland.

[††††]"FreeStyle" is a trademark of Abbott Diabetes Care, Ltd.. UK)

Enzymatic spectrophotometric absorbance reference method

An enzymatic spectrophotometric absorbance (EZA) assay was used as a reference method to quantify lactose in model and commercial ingredient dilutions. Commercial lactose/D-galactose enzyme assay kits (Catalog no. 10 176 303 035, R-Biopharm AG, Darmstadt, Germany) provided all necessary reagents to carry out the analysis. Instructions provided with the kits detailed assay preparation and lactose calculations, with minor adaptations being made to the procedure so that calculations could be done by weight basis as recommended by Lynch and Barbano (2007). A DU-650 UV-VIS spectrophotometer (Beckman Coulter, Brea, CA) capable of sequential single-beam absorbance analyses on up to 5 samples plus 1 blank per analysis session was used to measure the absorbance of prepared assays at an analysis wavelength of 340 nm. Disposable-type acrylic cuvettes with a 1 cm pathlength were used. Each sample tested was measured in duplicate, with all results reported as the average of duplicates.

Sample preparation for the enzymatic spectrophotometric absorbance assay. All samples were first diluted in DI water to ~ 0.05% w/w lactose. Solutions containing only lactose or low amounts of whey solids (≤ 3%) were filtered through 0.45 um nylon syringe filters and stored between 1 - 4 °C for later use in the EZA assay. Samples containing casein proteins were prepared in a similar fashion, except these solutions were centrifuged at 2,097 x g for 10 mins (Savant high speed microcentrifuge HSC10K, Thermo Scientific, Waltham MA) to remove most of the casein from the suspensions prior to filtering. Samples containing higher amounts of whey solids (<27%) were impossible to filter through syringe filters due to their viscosity; these solutions were first clarified via centrifugation at 8410 x g for 10 minutes and then filtered through 0.2 um nylon centrifuge filters with a 2 mL capacity at 5000 x g for 15 minutes (Legend XTR, Thermo Scientific, Waltham MA)

Phase 1

The effect of solution pH and lactose concentration on BGM output bias was investigated in a 2x4 factorial experiment for 4 different BGM brands. Eight model lactose solutions were created by combining pH 6.94, 6.71, 6.35, or 6.05 PBS buffers with lactose stock solutions to create 0.2% and 0.4% lactose versions of each buffer. The solutions were measured in a random order, in triplicate, using the proposed BGM method with 1 lot of test strips for each meter. The pH of each solution was remeasured after assay preparation to ensure that great changes in buffer pH did not occur. The BGM output values were compared to the theoretical glucose content of each solution (lactose content divided by 2) to calculate a percent bias difference for each reading. The PROC GLM procedure in SAS (Studio version, SAS Institute Inc., Cary, NC) was used to perform a two-way ANOVA analysis for each meter separately, treating pH and lactose concentration as class variables. Significant differences ($P < 0.05$) between experiment levels were detected using Tukey's Honest Significant Difference adjusted t-tests.

Phase 2

The effect of added non-lactose total solids and dairy ingredient type on BGM output was investigated in a 2x5 factorial experiment for 4 different BGM brands. Model WD or SMD solutions of 5 added solids levels (0, 2, 4, 6, or 8%) were spiked with a glucose monohydrate stock solution (Archer Daniels Midland, Chicago, IL) to create a constant 0.2% glucose content directly measurable by BGM biosensors. Whey protein isolate or micellar casein of ≥88% dry-basis protein was used to create the WD or SMD solutions respectively due to their individual purities in the primary non-lactose component in dairy ingredients, namely protein. Each solution was measured in a random order, in triplicate, using a slight modification of the proposed BGM method (0.1g DI water added instead of 0.1g lactase enzyme) with 1 lot of test

strips for each meter. The total solids content of each fully prepared dilution was verified using a Mark 3 LTE moisture analyzer (Sartorius AG, Goettingen, Germany). A percent bias difference for each reading was calculated between the BGM output values and known glucose content of each solution. The PROC GLM procedure in SAS was used to perform a two-way ANOVA analysis for each meter separately, treating ingredient type and added solids target as class variables.

Phase 3

Phase 3 was designed to assess the accuracy of a proposed calibration procedure for lactose analysis in dairy ingredients. To do this, model ingredient dilutions were created that simulate a dilution scheme representing the range of lactose contents found in common commercial dairy ingredients while also respecting the analytical range of the BGM brands tested. Representing WD ingredient model calibration standards were 3 different total solids categories (0.5, 3.0, or 27.0%) of 6 equally spaced lactose concentrations (0.10-0.62% lactose for the 0.5% and 3.0% WD solids categories; 0.08-0.44% lactose for the 27% WD solids category). SMD model solutions were prepared using similar specifications, except the 27.0% level was omitted due to viscosity concerns associated with SMD ingredient presence at this solids level. Each solution was measured in a random order in duplicate using the proposed BGM method with 3 different test strip lots for each the Nova Max Plus and FreeStyle Precision Neo meters. Each solution was also measured using the EZA reference method, and the paired BGM-EZA results were combined to create linear calibration curves specific for each solids combination, BGM brand, and BGM test strip lot. To verify the calibration curves, 6 model dilutions covering a similar range of lactose contents and solids combinations (0.12-0.58% lactose for the 0.5% and 3.0% WD and SMD solids categories; 0.09-0.44% lactose for the 27% WD solids category) were

prepared independently from each model calibration standard described earlier. The independent verification samples were measured using the BGM and EZA reference methods, with the BGM results now being converted to percent lactose using equation 4-1. Method comparison statistics were calculated for the paired BGM-EZA verification sample results using the PROC REG procedure in SAS to determine confidence intervals (α=0.05) for the linear slope and intercept parameters. Excel (Microsoft, Redmond, WA) was used to calculate average absolute percent bias difference and average bias difference between each paired BGM and EZA verification result as well. A coefficient of variation was also calculated between duplicate BGM measurements, with results expressed as an average result for all 3 test strip lots used.

Equation 4-1 Equation to convert blood glucose meter readings (in mg/dL glucose) to percent lactose based off of linear calibration parameters (slope and intercept) and dilution factors used at each step of sample preparation (F_i).

$$\%\ \mathbf{Lactose} = \frac{\mathbf{meter\ reading - intercept}}{\mathbf{slope} * \mathbf{F_i} * \mathbf{1000}}$$

Where F_i= dilution factor #1 *dilution factor #2*…..*dilution factor #i.

Phase 4

Lactose in commercial dairy ingredient samples was measured in duplicate using the proposed BGM method and EZA reference method. Two lots of test strips were used for each the Nova Max Plus and FreeStyle Precision Neo BGM brands. The same dilution schemes and linear calibration curve parameters established in phase 3 were used to quantify lactose in phase 4. Excel was used to calculate absolute percent bias difference and bias difference between each paired BGM-EZA lactose result, and a coefficient of variation was calculated between duplicate BGM measurements.

Results and Discussion

Phase 1

Table 4-1 details the impact that pH and lactose concentration may have on BGM output bias, expressed as percent bias difference (D) between the theoretical glucose content of the incubated solutions and what the meters displayed. The addition of lactase and lactose did not alter the buffer pH greatly (≤ 0.08 pH unit shift after addition).

Table 4-1 The effect of sample solution pH and lactose concentration on percent bias difference (D) between the expected and measured glucose content for 4 different blood glucose meter (BGM) brands.

	D (%)[1]							
	Nova Max plus meter		FreeStyle Precision Neo meter		OneTouch Verio flex meter		ReliOn Prime meter	
pH	0.2% lactose	0.4% lactose	0.2% lactose	0.4% lactose	0.2% lactose	0.4% lactose	0.2% lactose	0.4% lactose
6.94	-14.83 ± 0.56^{a,x}	-14.61 ± 2.36^{a,x}	24.83 ± 5.07^{a,x}	32.45 ± 3.76^{a,x}	2.40 ± 1.95^{a,x}	-4.60 ± 1.76^{a,x}	48.23 ± 2.58^{a,x}	89.14 ± 4.00^{a,y}
6.71	-8.37 ± 6.36^{a,x}	-16.45 ± 2.27^{a,x}	27.89 ± 7.49^{a,x}	32.28 ± 0.77^{a,x}	3.50 ± 4.98^{a,x}	-11.93 ± 1.76^{a,y}	43.38 ± 0.00^{a,x}	91.89 ± 1.74^{a,y}
6.35	-16.80 ± 2.44^{a,x}	-20.10 ± 5.0^{a,x}	16.55 ± 0.97^{a,x}	24.63 ± 10.20^{a,x}	-7.41 ± 4.38^{b,x}	-22.95 ± 2.90^{b,y}	27.88 ± 2.44^{b,x}	74.71 ± 8.29^{b,y}
6.05	-30.56 ± 2.59^{b,x}	-30.88 ± 3.07^{b,x}	-15.23 ± 2.04^{b,x}	0.59 ± 3.80^{b,y}	-32.19 ± 0.56^{c,x}	-35.06 ± 4.67^{c,x}	-21.43 ± 2.04^{c,x}	15.32 ± 3.27^{c,y}

[1] $D = \frac{A-B}{B} \times 100\%$, where A is a given BGM reading and B is the theoretical glucose content for a given solution. Values are means ±S.D. N=3.

[a-c] D within a column with a different superscript differ significantly ($P < 0.05$)

[x-y] D within a row within a BGM type with a different superscript differ significantly ($P < 0.05$)

Effect of lactose concentration. At a given pH level for some BGM brands, lactose concentration can have a significant impact on BGM output bias as observed by noncomparable ($P<0.05$) percent bias differences between the two lactose levels tested. Two of the meters tested (ReliOn Prime and FreeStyle Precision Neo) showed a positive bias associated with lactose concentration, while the OneTouch Verio flex showed a negative bias. The Nova Max Plus meter was unique, showing no discernable ($P >0.05$) lactose-proportional bias at any of the pH levels tested. Since the BGM output biases are non-zero and dependent on lactose concentration for most meters, any BGM will need to have its output calibrated over a range of lactose concentrations to make useful quantifications. Calibration of the meter output being necessary is consistent with other literature where the BGM method has been used for lactose quantification (Amamcharla & Metzger, 2011; Heinzerling et al., 2012;), and is considered a draw-back to using the BGM method for lactose analysis in dairy products (Churakova et al., 2019).

Effect of pH. Without exception, the BGM output biases were found to be comparable ($P >0.05$) between the 6.94 and 6.71 pH levels within a lactose level and BGM brand. Conversely, the lowest pH level (6.05) always registered a significantly ($P <0.05$) lower BGM output bias than any other pH level within a lactose level and BGM brand. This is consistent with reports showing BGM signal depression when performing glucose measurements in blood at pH< 6.8 due to a drop in the activity level for the mediator enzymes in a test strip (Dungan et al., 2007). A reduction in lactase activity at the solution incubation step of the BGM method, which would effectively lower the amount of glucose in solution, is not expected to occur at the pH values used here given that the lactase used has a pH-dependent rate maximum between pH 6 and 7. The authors chose to keep all further measurement solutions in this work above pH=6.7 when possible.

Phase 2

Table 4-2 details the statistical impact that different amounts or types of dairy ingredients can have on a given BGM brands output bias, with bias being expressed as the percent bias difference (D) between the fixed 0.2% glucose content of each solution and what the meters output. Figure 4-1 graphically illustrates this same impact for each individual model dilution analyzed.

Table 4-2 ANOVA summary detailing the effect of skim milk- or whey derived solids at different addition rates on percent bias difference (D) between expected and measured glucose values for 4 different blood glucose meter brands at a fixed glucose concentration of 0.2%.

		Nova Max Plus meter		FreeStyle Precision Neo meter		One Touch Verio Flex Meter		ReliOn Prime meter	
Error Source	DF	Error SS[1]	p-value	Error SS	p-value	Error SS	P-value	Error SS	P-value
Ingredient type	1	48.25	0.0317*	303.6	<0.0001*	68.39	0.0116*	40.68	0.2047
Added solids amount	4	255.9	0.0011*	1066	<0.0001*	2257	<0.0001*	1956	<0.0001*
Type x Amount	4	79.62	0.1056	161.1	0.0129*	34.68	0.4414	91.48	0.4477
Model Error	9	388.5		1530		2361		2088	
Total Error	29	559.0		1724		2538		2562	

[1] SS stands for Sum of Squares, or the squared residuals of each category's contribution to the ANOVA model error.
* P-values < 0.05 are statistically significant.

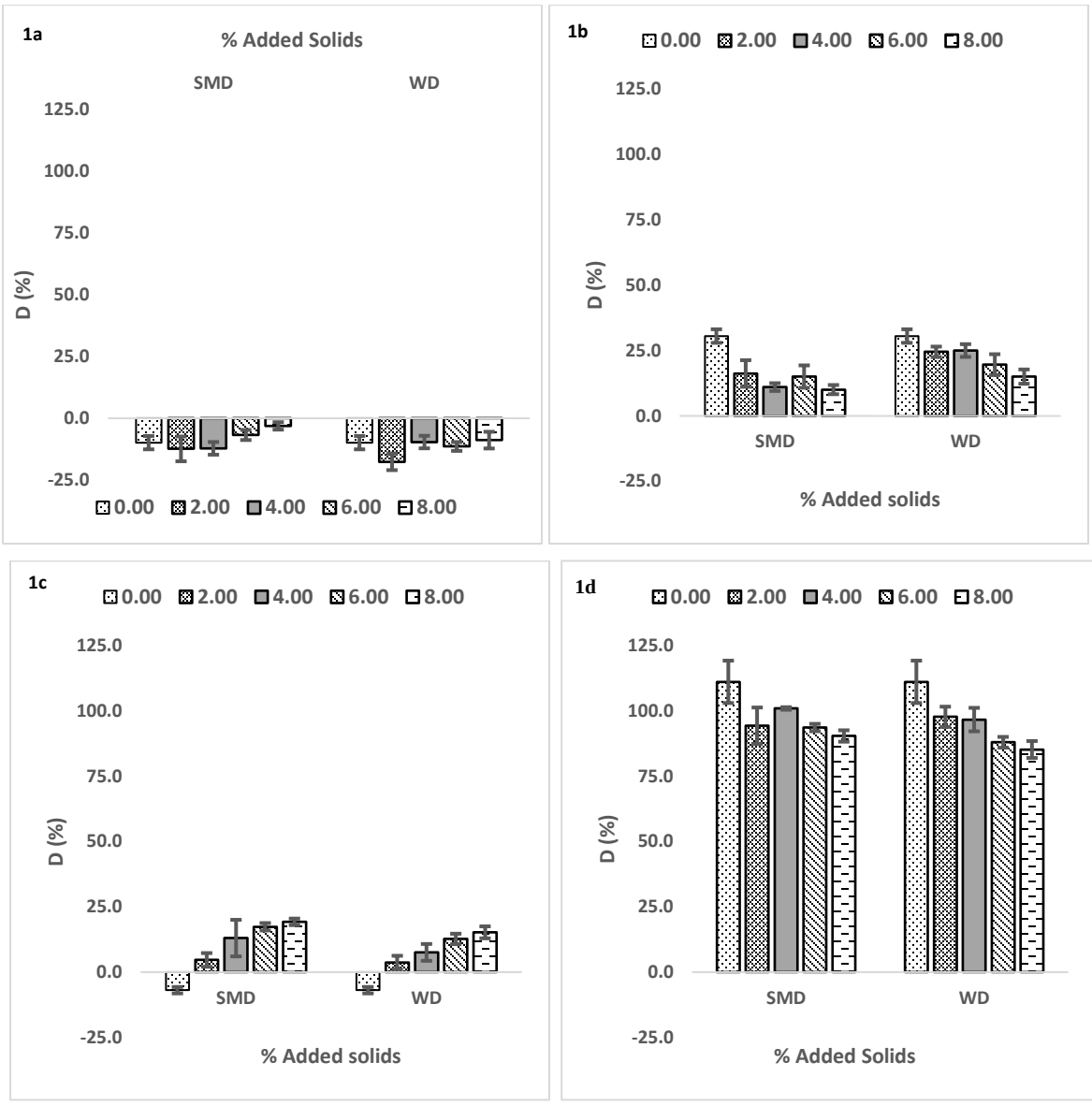


Figure 4-1 Effect of skim milk- or whey derived (SMD or WD) solids at different addition rates on percent bias difference (D) between expected and measured glucose values at a fixed glucose concentration of 0.2%. Figures 1a, 1b, 1c, and 1d respectively represent results for the Nova Max Plus, FreeStyle Precision Neo, OneTouch Verio Flex, and ReliOn Prime blood glucose meters. D = $\frac{\mathbf{A-B}}{\mathbf{B}} \times \mathbf{100}$%, where A is a given BGM reading and B is the spiked glucose concentration known for each test solution. Values are means; error bars are S.D. N=3.

Effect of ingredient type. All meter output biases except those for the ReliOn Prime meter were significantly ($P<0.05$) affected by ingredient type. However, studying figure 4-1 indicates that the differences between similar-solids WD- and SMD- entries are small on an individual basis, and it is worth noting that ingredient type contributed much less to the ANOVA model error than did added solids amount (on average for all meters, 9.28% and 81.22% of the model error is coming from solids type and amount respectively). It is also impossible to ascertain whether the differences seen due to ingredient type are truly due to the differences in ingredient character, or slight differences in actual total solids content measured between corresponding WD and SMD dilution series (data not shown). All the same, it may conservatively be best practice to account for solids type during calibration and subsequent quantifications.

Effect of added solids amount. All of the meter output biases were significantly ($P<0.05$) affected by added solids amount. As detailed in figure 4-1, increasing levels of solids for either ingredient type tested resulted in signal depression (decreasing D) for the FreeStyle Precision Neo meter and ReliOn Prime BGM brands, or signal elevation (increasing D) for the Nova Max Plus and OneTouch Verio Flex BGM brands. The reasons why may relate to the analogous hematocrit effect in blood, where increasing levels of non-lactose solids may hinder glucose from getting to the enzyme test site. Intuitively, this explanation should universally translate into decreased BGM output; however, the Nova Max Plus and OneTouch Verio flex meters are both known to algorithmically adjust their outputs for hematocrit when measuring glucose in blood (Ramljak et al., 2013), so it is theorized that the positive relationship between D and solids level observed for these meters is a result of the correction algorithm overcompensating for the presence of dairy ingredients. These results imply that the solids level

of an anticipated ingredient dilution must be accounted for during calibration. The most common commercial dairy ingredients were therefore partitioned into 3 dilution schemes that control the total solids inputted into a dilution while simultaneously keeping glucose concentrations between 0.03-0.3% post-lactose hydrolysis, well within the measurable range for most BGM brands. To model one possible generalized dilution scheme for most common dairy ingredients, model solutions of 0.5, 3.0, and 27% total solids were chosen to represent measurement-ready dilutions for 31-100%, 3.5-18, and <2.0% lactose dry-basis ingredients respectively. Assuming a moisture content of 4% for most dairy powders, the 0.5, 3.0, and 27.0% total solids targets translate into dilution factors of 0.0053, 0.0327, and 0.2857 respectively.

Best overall meter for lactose quantification in dairy ingredients. The Nova Max Plus meter was selected for further testing due to it's overall low and relatively stable bias over the range of lactose and solids levels tested. The FreeStyle Precision Neo was also selected for further testing, but for the opposite reason; it's poor performance in phases 1 & 2 serve as a comparison to the Nova Max Plus to verify that screening tests such as those performed to this point are relevant.

Phase 3

The calibration data is not shown but was of high quality. For example, none of the calibration curves had an r^2-value less than 0.986, indicating good linearity. Also, a limit of detection (LOD) was calculated for each calibration curve and test strip lot used (LOD=3*RMSE/slope, where RMSE is the root mean square error for a particular model) and averaged 0.037% (0.024%-0.060%) lactose for the Nova Max Plus meter or 0.051% (0.025-0.062%) lactose for the FreeStyle Precision Neo, all of which of which are well below the lactose levels measured in this work. Also, it was determined from the calibration data that test strip lot-

specific calibration parameters should be used for subsequent lactose quantifications as evidenced by non-comparable ($P < 0.05$) slope parameters between some of the test strip lots used in the higher solid's samples.

The paired method statistics between the EZA reference method and calibration adjusted BGM method lactose results are shown in tables 4-3 and 4-4 for the independently prepared verification WD and SMD model dilutions respectively.

Table 4-3 Method comparison statistics between the blood glucose meter (BGM) method and enzymatic spectrophotometric absorbance (EZA) reference method for lactose measurement using BGM strip lot-specific calibration parameters for model whey derived (WD) verification dilutions.

Dilution model	Parameter	Nova Max Plus			FreeStyle Precision Neo		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
0.5% WD	Slope	1.008	0.965	0.953	0.988	0.989	1.037
	Intercept	0.002	0.018	0.025	0.003	0.009	0.000
	r^2	0.990	0.978	0.986	0.977	0.990	0.990
	95% CI Slope	0.935 to 1.080	0.862 to 1.068	0.875 to 1.036	0.880 to 1.096	0.918 to 1.061	0.959 to 1.116
	95% CI Intercept	-0.025 to 0.028	-0.021 to 0.056	-0.006 to 0.055	-0.037 to 0.044	-0.018 to 0.036	-0.029 to 0.030
	d[1]	0.00 (-0.03 to 0.02)	0.01 (-0.03 to 0.04)	0.01 (-0.02 to 0.05)	0.00 (-0.03 to 0.04)	0.01 (-0.01 to 0.04)	0.01 (-0.01 to 0.05)
	AD[2]	5.69 (0.49 to 19.5)	6.23 (1.09 to 13.9)	6.87 (1.18 to 22.7)	5.45 (1.02 to 11.4)	4.60 (0.14 to 13.5)	4.64 (0.55 to 14.0)
	Average % CV[3]		2.75 (0.35 to 6.60)			2.84 (0.00 to 7.08)	
3.0% WD	Slope	1.029	1.050	1.006	1.032	1.082*	0.960
	Intercept	-0.001	0.001	0.006	-0.007	-0.018	0.003
	r^2	0.996	0.990	0.991	0.993	0.995	0.989
	95% CI Slope	0.983 to 1.075	0.973 to 1.126	0.937 to 1.076	0.973 to 1.092	1.025 to 1.139	0.890 to 1.030
	95% CI Intercept	-0.019 to 0.016	-0.027 to 0.030	-0.021 to 0.032	-0.030 to 0.015	-0.039 to 0.003	-0.023 to 0.030
	d[1]	0.01 (-0.01 to 0.02)	0.02 (-0.01 to 0.04)	0.01 (-0.02 to 0.03)	0.00 (-0.02 to 0.02)	0.01 (-0.02 to 0.03)	-0.01(-0.04 to 0.02)
	AD[2]	3.29 (0.18 to 11.5)	6.16 (0.48 to 13.0)	5.76 (0.07 to 24.1)	3.68 (0.28 to 13.5)	5.87 (1.08 to 15.7)	5.10 (0.87 to 11.7)
	Average % CV[3]		3.00 (0.00 to 9.27)			2.66 (0.48 to 8.10)	
27% WD	Slope	0.985	0.961*	1.035	1.045	1.019	0.984
	Intercept	0.004	0.009*	-0.007	-0.007	0.000	-0.001
	r^2	0.997	0.998	0.995	0.986	0.986	0.984
	95% CI Slope	0.946 to 1.023	0.931 to 0.992	0.983 to 1.087	0.958 to 1.131	0.931 to 1.106	0.887 to 1.064
	95% CI Intercept	-0.006 to 0.015	0.000 to 0.0172	-0.007 to 0.006	-0.030 to 0.017	-0.024 to 0.024	-0.025 to 0.023
	d[1]	0.00 (-0.01 to 0.01)	0.00 (-0.01 to 0.01)	0.00 (-0.02 to 0.02)	0.00 (-0.02 to 0.03)	0.00 (-0.02 to 0.04)	-0.01(-0.04 to 0.02)
	AD[2]	3.84 (0.02 to 10.8)	2.97 (0.43 to 11.3)	5.46 (0.73 to 18.0)	5.16 (0.33 to 11.1)	4.47 (0.38 to 12.0)	7.32 (0.59 to 16.1)
	Average % CV[3]		2.01 (0.31 to 11.20)			4.93 (0.00 to 10.84)	

[1] d=A-B, where A is the average BGM method result, and B is the average EZA reference method result. Numbers in parentheses are a range. Units are % lactose.

[2] AD= $\frac{|A-B|}{B} \times 100\%$, where A is the average BGM method result, and B is the average EZA reference method result. Numbers in parentheses are a range. Units are % lactose.

[3] Average %CV calculated by averaging individual BGM %CV results for a given blood glucose meter and model dilution set.

* Statistical difference ($P<0.05$) from 1 or 0 for the slope or intercept parameters respectively.

Table 4-4 Method comparison statistics between the blood glucose meter (BGM) method and enzymatic spectrophotometric absorbance (EZA) reference method for lactose measurement using BGM strip lot-specific calibration parameters for model skim milk derived (SMD) verification dilutions.

Dilution model	Parameter	Nova Max Plus			FreeStyle Precision Neo		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
0.5% SMD	Slope	1.011	1.021	0.990	0.958	1.080*	1.030
	Intercept	-0.002	-0.002	0.010	0.018	-0.024	-0.007
	r^2	0.994	0.996	0.996	0.986	0.992	0.993
	95% CI Slope	0.956 to 1.067	0.976 to 1.065	0.945 to 1.035	0.878 to 1.039	1.009 to 1.151	0.971 to 1.089
	95% CI Intercept	-0.023 to 0.018	-0.018 to 0.015	-0.007 to 0.027	-0.012 to 0.048	-0.050 to 0.002	-0.029 to 0.015
	d[1]	0.00 (-0.02 to 0.03)	0.01 (-0.01 to 0.02)	0.01 (-0.01 to 0.02)	0.00 (-0.02 to 0.03)	0.00 (-0.02 to 0.05)	0.00 (-0.02 to 0.03)
	AD[2]	2.17 (0.09 to 8.23)	2.95 (0.07 to 6.89)	3.64 (0.01 to 10.23)	4.55 (0.72 to 9.95)	5.69 (1.18 to 14.7)	4.69 (0.40 to 12.9)
	Average % CV[3]		2.45 (0.00 to 6.14)			2.75 (0.00 to 8.48)	
3.0% SMD	Slope	0.926*	0.967	1.003	1.039 *	0.961	1.033
	Intercept	0.020*	0.013	0.009	-0.012	0.011	0.002
	r^2	0.997	0.993	0.997	0.998	0.995	0.997
	95% CI Slope	0.890 to 0.963	0.909 to 1.025	0.965 to 1.040	1.006 to 1.072	0.911 to 1.012	0.993 to 1.073
	95% CI Intercept	0.007 to 0.034	-0.008 to 0.035	-0.005 to 0.022	-0.024 to 0.000	-0.007 to 0.030	-0.013 to 0.017
	d[1]	0.00 (-0.02 to 0.02)	0.00 (-0.03 to 0.02)	0.01 (0.00 to 0.02)	0.00 (-0.01 to 0.02)	0.01 (-0.01 to 0.02)	0.00 (-0.01 to 0.01)
	AD[2]	4.78 (0.15 to 21.7)	3.81 (0.30 to 10.9)	4.09 (0.03 to 19.7)	2.47 (0.42 to 7.47)	4.56 (0.10 to 8.90)	3.14 (0.77 to 9.73)
	Average % CV		1.47 (0.00 to 4.68)			2.10 (0.30 to 9.71)	

[1] d=A-B, where A is the average BGM method result, and B is the average EZA reference method result. Numbers in parentheses are a range. Units are % lactose.

[2] AD= $\frac{|A-B|}{B} \times 100\%$, where A is the average BGM method result, and B is the average EZA reference method result. Numbers in parentheses are a range. Units are % lactose.

[3] Average %CV calculated by averaging individual BGM %CV results for a given blood glucose meter and model dilution set.

* Statistical difference ($P<0.05$) from 1 or 0 for the slope or intercept parameters respectively.

Whey derived ingredient dilutions. The BGM method precision is acceptable given an average %CV ≤ 4.93%. Accuracy in terms of agreement between the proposed BGM and EZA reference method was also good, with only 2 instances where regression slopes were significantly different from 1 (3.0% WD model dilutions, FreeStyle Precision Neo, strip lot 2; 27 % WD model dilutions, Nova Max Plus, strip lot 2) and only 1 instance where intercepts were significantly different from 0 (27 % WD model dilutions, Nova Max Plus, strip lot 2). A slope and intercept not statistically different ($P>0.05$) from 1 or 0 indicate good agreement between the two methods. Average difference (d) in lactose results between the methods was low, with the average $|d| \leq 0.02\%$ lactose. Also, d did not trend high or low for any of the WD model dilutions, test strip lots, or meters tested, indicating that any differences observed between the test and reference methods were random. Examining the absolute percent bias difference (AD) still invokes a favorable bias comparison between the BGM and EZA reference method given an average AD between 2.97% and 7.32%. However, examining the range of AD for each average listed in table 4-3 reveals some relatively high values; the highest absolute percent bias differences (AD>15%) are universally associated with the lowest lactose concentrations measured (<0.12% lactose). It is expected that there is a consistent amount of random error associated with the BGM measurement that is low overall (as indicated by $|d| <0.02\%$ lactose), but relatively high on a percent difference basis when these errors occur at lower lactose concentrations.

Skim-milk derived ingredient dilutions. An upper-limit to the lactose content readable by the Nova Max Plus was observed early on, and the calibration range had to be adjusted down from 0.61% to 0.58% lactose. This was observed as a meter output that would not increase above 260 mg/dL glucose regardless of increasing lactose concentration, likely due to hinderance

of the BGM enzyme test site by the large casein proteins. Beyond that, interpretations of the SMD model dilutions results largely mirror those of the WD model dilutions. It can be concluded that the proposed dilution and ingredient type- based calibration schemes are likely to be successful at accurately predicting the lactose content of commercial dairy ingredient samples.

Phase 4

The as-is lactose contents of 15 dry commercial dairy ingredients measured using the EZA reference method and proposed BGM method with the modified calibration procedure are shown in tables 4-5 and 4-6 for the Nova Max Plus and FreeStyle Precision Neo meter respectively. The BGM method's precision was acceptable for both the Nova Max Plus and FreeStyle Precision Neo meter, with average %CV <2.83% and <3.54% respectively. Accuracy of the BGM method in terms of agreement between the BGM results and EZA results was found to depend on the meter used.

Table 4-5 Comparison of the blood glucose meter (BGM) method and enzymatic spectrophotometric absorbance (EZA) reference method for lactose measurement in commercial dairy ingredient samples using BGM strip lot-specific calibration parameters for the Nova Max Plus BGM**.**

	Lactose Concentration (%)								
		BGM method		CV (%)[1]		d (%)[2]		AD (%)[3]	
Ingredient	EZA	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
Milk Permeate	81.9	81.1	83.3	1.75	0.42	-0.78	1.41	0.96	1.72
NFDM LH	51.4	49.2	51.3	6.46	2.72	-2.20	-0.09	4.29	0.18
NFDM HH	49.6	49.6	50.7	7.14	4.83	-0.01	1.12	0.01	2.26
Buttermilk	47.2	44.7	45.4	0.79	1.54	-2.55	-1.86	5.39	3.93
MPC-70	12.9	12.7	13.6	0.78	9.81	-0.16	0.70	1.23	5.46
MPI-85	5.78	6.18	5.91	0.79	3.91	0.40	0.13	6.89	2.33
Miceller Casein	4.37	5.14	4.83	2.87	1.40	0.77	0.46	17.6	10.6
Whey Permeate	76.3	71.2	71.1	1.91	1.82	-5.18	-5.28	6.78	6.91
Whey	68.8	69.6	69.3	1.46	2.79	0.84	0.57	1.22	0.83
WPC 34	49.1	45.0	47.2	1.50	0.68	-4.17	-1.93	8.48	3.92
WPC 80	5.90	5.59	5.99	0.00	2.58	-0.31	0.09	5.22	1.59
WPI #1	1.47	1.41	1.44	3.65	3.57	-0.05	-0.04	3.65	3.02
WPI #2	0.03	<0.04	<0.05	-	-	-	-	-	-
WPI #3	0.01	<0.04	<0.05	-	-	-	-	-	-
WPI #4	0.70	0.68	0.67	4.21	0.70	-0.02	-0.02	3.05	3.54
Averages				2.56	2.83	-1.03	-0.36	4.98	3.56

[1] %CV calculated between duplicate BGM measurements for each test strip lot. N=2

[2] d=A-B, where A is the average BGM method result, and B is the average EZA reference method result.

[3] AD= $\frac{|A-B|}{B} \times 100\%$, where A is the average BGM method result, and B is the average EZA reference method result.

Table 4-6 Comparison of the blood glucose meter (BGM) method and enzymatic spectrophotometric absorbance (EZA) reference method for lactose measurement in commercial dairy ingredient samples using BGM strip lot-specific calibration parameters for the FreeStyle Precision Neo BGM.

Ingredient	Lactose Concentration (%)			CV (%)[1]		d (%)[2]		AD (%)[3]	
		BGM method							
	EZA	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
Milk Permeate	81.9	87.4	95.8	4.25	5.56	5.56	13.89	6.79	17.0
NFDM LH	51.4	54.3	60.9	4.01	1.98	2.98	9.50	5.81	18.5
NFDM HH	49.6	52.0	54.7	1.68	4.42	2.47	5.15	4.98	10.4
Buttermilk	47.2	51.3	50.6	0.85	2.38	4.05	3.41	8.57	7.23
MPC-70	12.9	13.1	14.6	1.65	2.08	0.22	1.67	1.67	13.0
MPI-85	5.78	5.84	6.24	2.46	8.46	0.06	0.46	1.05	7.91
Micellar Casein	4.37	4.27	4.32	6.73	3.49	-0.10	-0.05	2.32	1.15
Whey Permeate	76.3	74.9	78.6	2.47	0.82	-1.41	2.28	1.85	2.99
Whey	68.8	65.7	67.8	3.74	3.78	-3.04	-0.97	4.42	1.42
WPC 34	49.1	49.1	49.2	2.08	1.30	-0.06	0.05	0.12	0.11
WPC 80	5.90	5.44	5.74	1.98	0.68	-0.32	-0.03	5.37	0.44
WPI #1	1.47	1.34	1.68	1.60	1.28	-0.13	0.21	9.13	14.0
WPI #2	0.03	<0.04	<0.05	-	-	-	-	-	-
WPI #3	0.01	<0.04	<0.05	-	-	-	-	-	-
WPI #4	0.70	0.82	0.92	1.75	9.80	0.12	0.23	17.1	32.3
Averages				2.71	3.54	0.80	2.75	5.32	9.73

[1] %CV calculated between duplicate BGM measurements for each test strip lot. N=2

[2] d=A-B, where A is the average BGM method result, and B is the average EZA reference method result.

[3] AD= $\frac{|A-B|}{B} \times 100\%$, where A is the average BGM method result, and B is the average EZA reference method result.

Nova Max Plus accuracy. The Nova Max Plus BGM achieved relatively accurate results, with an average AD of 4.98% or 3.56% for test strip lots 1 or 2 respectively. One outlier was noted in the Nova Max Plus results when measuring lactose in micellar casein (AD= 17.58% and AD=10.60% for test strip lots 1 and 2 respectively). The high error in terms of AD for micellar casein is consistent with observations in phase 3 where random BGM measurement error is inflated on a percent basis at lower lactose concentrations; micellar casein was relatively low in lactose for the 3.0% SMD dilution -specific calibration series, measuring 0.14% lactose via EZA method before adjusting for dilution (calibration range was 0.10-0.58% lactose). Thus, accurately measuring low amounts of lactose for a given calibration series may be a limitation of the BGM method. An alternative hypothesis for the high AD associated with this particular micellar casein ingredient could be due to glycosylation of the k-casein macropeptide portion of the protein. Glycosylated dairy proteins can be covalently linked to glucose, galactose (Recio et al., 2009) or lactose (Lillard et al., 2008), and the extent of glycosylation in k-casein macropeptide in particular can be difficult to predict since it depends largely on a cow's lactation cycle (O'Riordan et al., 2014); while it is unlikely that these protein-attached carbohydrates could cause measurement inaccuracies with the proposed BGM lactose analysis, it must be mentioned as a possibility since proving otherwise is beyond the scope of this work.

FreeStyle Precision Neo accuracy. The FreeStyle Precision Neo results showed a lower level of accuracy relative to the Nova Max Plus, with AD=5.32% or 9.73% for test strip lots 1 or 2 respectively. A plausible explanation for the large amount of seemingly random error associated with the FreeStyle Precision Neo may relate to observations in phase 2, where the FreeStyle Precision Neo was the only BGM with an output bias significantly ($P<0.05$) affected by the interaction between the type and amount of added total solids (see table 4-2). The

FreeStyle Precision Neo showed acceptable accuracy in phase 3 when the SMD material used to create the calibration standards was the same as the independent verification samples measured, implying that poor performance of the FreeStyle Precision Neo with commercial samples may stem from material discrepancies between the commercial samples and the SMD ingredient chosen as background during calibration; this issue appears to be greatly exacerbated as higher levels of solids are incorporated (see high AD values associated with WPI #1 and #4 in table 4-6). Thus, the FreeStyle Precision Neo may not be flexible enough to measure lactose in many different kinds of materials using the same model calibration parameters, which limits its practical use.

Conclusions

BGM biosensors can be successfully adapted to measuring lactose in dairy ingredients if the following key concepts are adhered to:

1.) 1X PBS buffer adjusted to pH 7 should be used in dilutions as needed in order to control the final dilution pH to between 6.7-7.0.

2.) Dilution schemes and corresponding calibration standards specific for a select range of dairy ingredients should be used. The composition of the material used in the calibration standards should loosely resemble the ingredients ultimately being measured (ie: SMD versus WD ingredients). Total diluted solids presence of 0.5, 3.0, and 27% can represent 31-100%, 3.5-18, and <2.0% expected dry-basis lactose ingredients respectively (these solids levels represent dilution factors of 0.005, 0.0327, or 0.2857 respectively for future commercial sample measurements). Pure lactose can be used to adjust the lactose concentration of the calibration standards anywhere from 0.08-0.6% as-is lactose.

3.) A practical application of the method will use a BGM that is accurate, precise, and flexible (the Nova Max Plus worked well here).

Using these suggestions, a BGM can be used to routinely measure lactose in dairy ingredients. The suggested dilution factors are relevant to most dried dairy ingredients, but different dilution factors can be adapted to other dairy ingredients outside the scope of this work (eg. liquid retentate) so long as the same concepts are applied.

Once calibration curves have been developed, the advantages of the BGM method include achieving results in about 20 minutes, requiring no hazardous chemicals or expensive instrumentation, and requiring no sample preparation outside of sample dilution. Also, the method is low cost enough for routine use given a single test cost of <$0.50 for the test strip and a one-time cost of ~$20 for the BGM. It is not recommended that the BGM method be used to verify lactose absence in foods since lactose is not quantified directly with the method; thus, this method is more useful for applications such as process optimization or design, ingredient standardization, product development, and routine lactose measurements for quality control purposes.

References

Amamcharla, J.K., and L.E. Metzger. 2011. Development of a rapid method for the measurement of lactose in milk using a blood glucose biosensor. J. Dairy Sci. 94:4800–4809. doi:10.3168/jds.2011-4416.

Booth, M.A., H. Karaosmanoglu, Y. Wu, and A. Partridge. 2017. Biosensor platforms for detecting target species in milk samples. M.U. Ahmed, M. Zourob, and E. Tamiya, ed. Royal Society of chemistry, Cambridge.

Churakova, E., K. Peri, J.S. Vis, D.W. Smith, J.M. Beam, M.P. Vijverberg, M.C. Stor, and R.T. Winter. 2019. Accurate analysis of residual lactose in low-lactose milk: comparing a variety of analytical techniques. Int. Dairy J.. doi:10.1016/j.idairyj.2019.02.020.

Conzuelo, F., A.J.A. Reviejo, and J.M.J. Pingarron. 2012. Lactose in milk and dairy products : A focus on biosensors. V. Preedy, ed. Royal Society of chemistry, Cambridge, U.K.

Dirienzo, D. 2016. Research gaps in the use of dairy ingredients in food aid products. Food Nutr. Bull. 37:S51–S57. doi:10.1177/0379572116628351.

Dungan, K., J. Chapman, S.S. Braithwaite, and J. Buse. 2007. Glucose measurement: Confounding issues in setting targets for inpatient management. Diabetes Care 30:403–409. doi:10.2337/dc06-1679.

Erbach, M., G. Freckmann, R. Hinzmann, B. Kulzer, R. Ziegler, L. Heinemann, and O. Schnell. 2016. Interferences and limitations in blood glucose self-testing: An overview of the current knowledge. J. Diabetes Sci. Technol. 10:1161–1168. doi:10.1177/1932296816641433.

Frias, J.P., C.G. Lim, J.M. Ellison, and C.M. Montandon. 2010. Review of adverse events associated with false glucose readings measured by GDH-PQQ-based glucose test strips in the presence of interfering sugars. Diabetes Care 33:728–729. doi:10.2337/dc09-1822.

Gabbanini, S., E. Lucchi, F. Guidugli, R. Matera, and L. Valgimigli. 2010. Anomeric discrimination and rapid analysis of underivatized lactose, maltose, and sucrose in vegetable matrices by U-HPLC - ESI-MS/MS using porous graphitic carbon. J. Mass Spectrom. 45:1012–1018. doi:10.1002/jms.1750.

Glithero, N., C. Clark, L. Gorton, W. Schuhmann, and N. Pasco. 2013. At-line measurement of lactose in dairy-processing plants. Anal. Bioanal. Chem. 405:3791–3799. doi:10.1007/s00216-012-6598-y.

Gulzar, M., and J.C. Jacquier. 2018. Impact of Residual Lactose on Dry Heat-Induced Pre-texturization of Whey Proteins. Food Bioprocess Technol. 11:1985–1994. doi:10.1007/s11947-018-2162-x.

Heinzerling, P., F. Schrader, and S. Schanze. 2012. Measurement of enzyme kinetics by use of a blood glucometer: Hydrolysis of sucrose and lactose. J. Chem. Educ. 89:1582–1586. doi:10.1021/ed200735f.

Huber, K.C., and J.N. BeMiller. 2017. Carbohyrdates. 5th ed. D. Srinivasan and K.L. Parkin, ed. CRC Press, Baton Rouge.

Huppertz, T., and I. Gazi. 2016. Lactose in dairy ingredients: Effect on processing and storage stability. J. Dairy Sci. 99:6842–6851. doi:10.3168/jds.2015-10033.

Lillard, J., Clare, D., Daubert, C. 2008. Glycosylation and expanded utility of a modified whey protein ingredient via carbohydrate conjugation at low pH. J. Dairy Sci. 92: 35-48. doi: 10.3168/jds.2008-1263.

Lynch, J., and D.M. Barbano. 2007. Determination of the lactose content of fluid milk by spectrophotometric enzymatic analysis using weight additions and path length adjustment: Collaborative study. J. Aoac Int. 90:196–216.

Margolies, B.J., and D.M. Barbano. 2017. Determination of fat, protein, moisture, and salt content of Cheddar cheese using mid-infrared transmittance spectroscopy. J. Dairy Sci. 101:924–933. doi:10.3168/jds.2017-13431.

McCarthy, K.S., M. Parker, A. Ameerally, S.L. Drake, and M.A. Drake. 2017. Drivers of choice for fluid milk versus plant-based alternatives: What are consumer perceptions of fluid milk?. J. Dairy Sci. 100:6125–6138. doi:10.3168/jds.2016-12519.

Olabi, A., S. Jinjarak, R. Jiménez-Flores, J.H. Walker, and H. Daroub. 2015. Compositional and sensory differences of products of sweet-cream and whey buttermilk produced by microfiltration, diafiltration, and supercritical CO2. J. Dairy Sci. 98:3590–3598. doi:10.3168/jds.2014-9141.

O'Riordan, N., Kane, M., Joshi, L., Hickey, R.M. 2014. Structural and functional characteristics of bovine milk protein glycosylation. Glycobiology. 24:220-236. doi:10.1093/glycob/cwt162

Paterson, A.H.J.H.J., and S.J.J. Kellam. 2009. Transformation of lactose for value-added ingredients. M. Corredig, ed. Woodhead Publishing, Boca Raton.

Ramljak, S., J.P. Lock, C. Schipper, P.B. Musholt, T. Forst, M. Lyon, and A. Pfützner. 2013. Hematocrit interference of blood glucose meters for patient self-measurement.. J. Diabetes Sci. Technol. 7:179–89. doi:10.1177/193229681300700123.

Recio, I., Moreno, F.J., Lopez-Fandino, R. 2009. Glycosylated dairy components: Their roles in nature and ways to make use of their biofunctionality in dairy products. M. Corredig, ed. Woodhead Publishing, Boca Raton.

Sansonetti, S., S. Curcio, V. Calabrò, and G. Iorio. 2010. Optimization of ricotta cheese whey (RCW) fermentation by response surface methodology. Bioresour. Technol. 101:9156–9162. doi:10.1016/j.biortech.2010.07.030.

Schleis, T.G. 2007. Interference of maltose, icodextrin, galactose, or xylose with some blood glucose monitoring systems.. Pharmacotherapy 27:1313–1321. doi:10.1592/phco.27.9.1313.

Sharma, S.K., and R.M. Leblanc. 2017. Biosensors based on β-galactosidase enzyme: Recent advances and perspectives. Anal. Biochem. 535:1–11. doi:10.1016/j.ab.2017.07.019.

Sluková, M., A. Hinková, S. Henke, F. Smrž, M. Lukačíková, V. Pour, and Z. Bubník. 2016. Cheese whey treated by membrane separation as a valuable ingredient for barley sourdough preparation. J. Food Eng. 172:38–47. doi:10.1016/j.jfoodeng.2015.03.023.

Upreti, P., L.L. McKay, and L.E. Metzger. 2006. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on Cheddar cheese quality: Changes in residual sugars and water-soluble organic acids during ripening. J. Dairy Sci. 89:429–443. doi:10.3168/jds.S0022-0302(06)72107-5.

Vanavanan, S., P. Santanirand, U. Chaichanajarernkul, A. Chittamma, J.A. DuBois, T. Shirey, and M. Heinz. 2010. Performance of a new interference-resistant glucose meter. Clin. Biochem. 43:186–192. doi:10.1016/j.clinbiochem.2009.09.010.

Chapter 5 - Conclusions

Lactose is an important functional and nutritional constituent of dairy ingredients. The amount of lactose present in a particular ingredient can have important consequences for it's shelf life, quality, and economic value. Having a rapid way to measure lactose in these ingredients could lead to improvements in their utility and quality, but current methods of lactose analysis used in the dairy industry are time consuming and expensive. There is a need for a low-cost and rapid method to routinely measure lactose in dairy ingredients, and this need could be met immediately with blood glucose meter (BGM) biosensors.

To develop the BGM method for lactose analysis in dairy ingredients, the procedure outlined by Amamcharla and Metzger (2011) for BGM-based lactose analysis in milk was modified. Due to dairy ingredient variability relative to milk, it was expected that modifications to the sample preparation and calibration procedures used for milk would be needed. Key findings in this work support that hypothesis: pH of the background measurement solution must be controlled to a level between 6.7 and 7 using buffer when possible. Different amounts and types of non-lactose solids present in the measurement background can create a bias in BGM measurements, so this bias must be accounted for during calibration by controlling the input solids level during sample dilution (dairy ingredients can be partitioned into 3 categories with target dilution factors of 0.0053, 0.0327, and 0.2857 for 31-100%, 3.5-18, and <2.0% lactose dry-basis ingredients respectively).

Once these modifications to the sample preparation and calibration procedure were made, accurate and precise measurements in model and commercial dairy ingredient dilutions were achieved. Analysis is simple, requires few tools, and a lactose result can be obtained in 20 minutes or less. A meter costs a one-time fee of ~$20, and each individual analysis costs ~$0.50

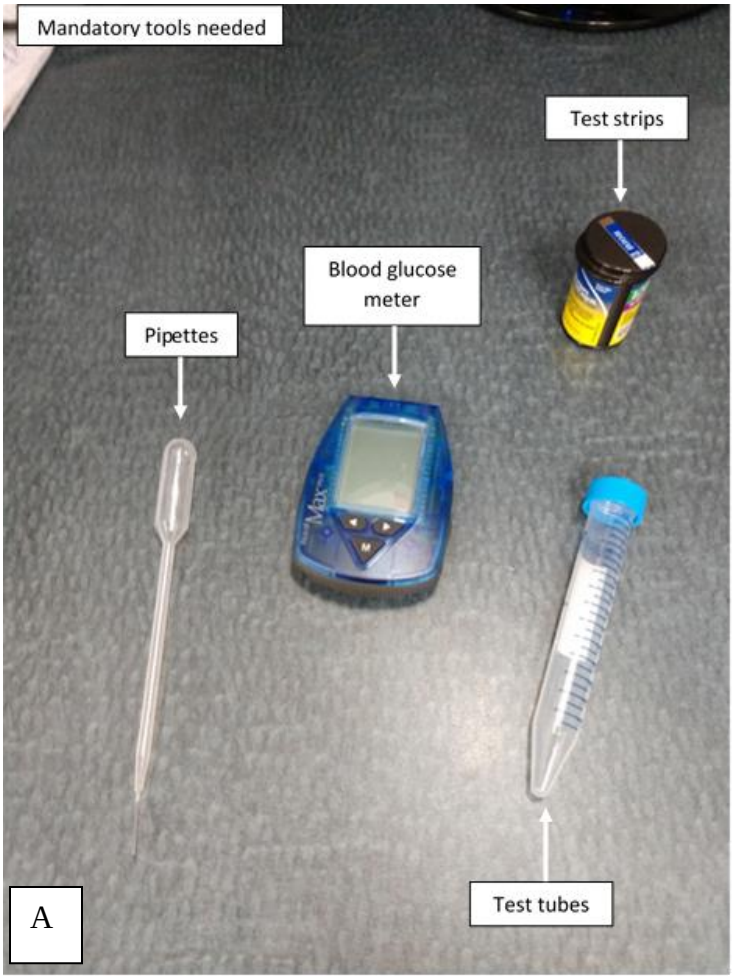
for the test strips, which compares very favorably with the pricing of commercial biosensors currently available.

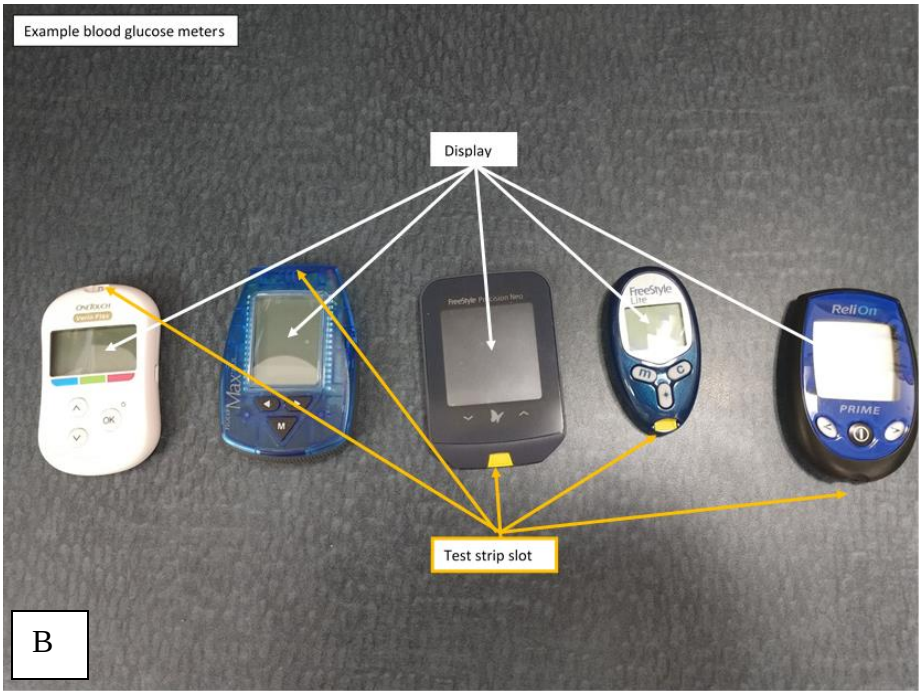
Future research with the BGM method of lactose measurement would involve measuring lactose in dairy foods. A similar procedure as that employed here for dairy ingredients could be extended to dairy foods since the same interferent-concepts apply. Theoretically, a calibration curve can be created for any dairy food or ingredient, and subsequent lactose quantifications could be made for that food based on that calibrations curve's linear parameters. This concept is dependent on using a blood glucose meter with the flexibility to make accurate quantifications under the difficult conditions associated with whole foods such as high solids and fat contents (the author recommends starting with the Nova Max Plus).

Overall, a low-cost, rapid, and easy to perform lactose analysis for dairy ingredients has been outlined here. When the proper BGM is selected, a user simply has to create calibration standards that loosely resemble the material matrix that they desire to make measurements in, measure lactose in those standards using both a reference method and the BGM method, and then quantify lactose in future BGM measurements by using the obtained calibration parameters. The BGM method is well-suited for internal testing of lactose in ingredients for the purposes of quality control, process and product development, and ingredient standardization.

Appendix A - Collecting and analyzing data for phases 1 & 2

General procedure and tools for measuring lactose with a blood glucose meter





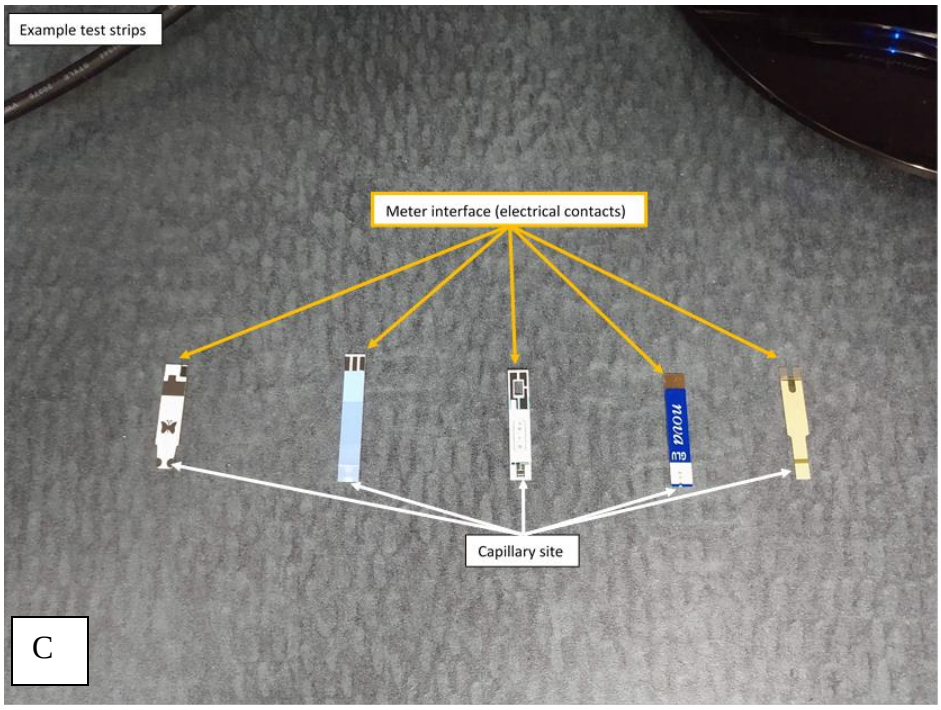


Figure A-1 Example tools needed to perform the BGM analysis method are shown in pane A. Major blood glucose meter sensor and test strip parts are shown in panes B and C respectively.

Table A-1 Blood glucose meter brands used in this work.

Meter Name	Enzyme-based detection element	Meter operating principle	Measurement range, mg/DL glucose	Sample volume (µL)	Suggested operating temperature (°C)	Suggested operating humidity (%)
ReliOn Prime	Glucose oxidase	Amperometry	20-600	0.5	10-40	20-80
FreeStyle Precision Neo	GDH-NAD	Amperometry	20-500	?	15-40	10-90
OneTouch Verio flex	GDH-FAD	Amperometry	20-600	0.4	10-40	10-90
Nova Max Plus	Glucose oxidase	?	20-600	0.3	14-40	10-90

BGM lactose measurement procedure:

1.) Disperse sample powders and/ or lactose in buffer to prescribed levels.

2.) Weight out 5.00g diluted samples into a test tube.

3.) Add 2% (~0.100g) lactase enzyme, and reweigh sample to confirm weight.

4.) Mix thoroughly.

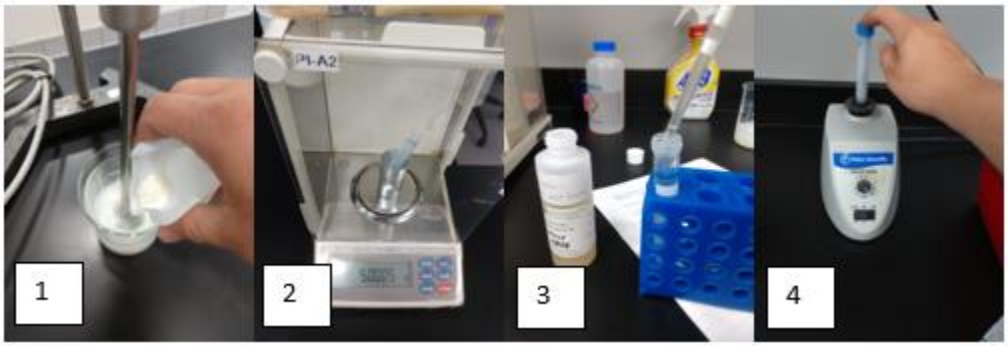


Figure A-2 Steps 1-4 of the blood glucose meter (BGM) lactose measurement procedure are shown visually from left to right, and are labeled for the corresponding step.

5.) Incubate sample in a water bath at 40 °C for >15 mins.

6.) Insert a new test strip into BGM.

7.) Pipette sample onto BGM capillary site.

8.) Record result (in mg/dL glucose). Convert glucose reading to percent lactose using equation 4-1.

9.) Measurements should be repeated in at least duplicate

Figure A-3 Steps 6-8 of the basic blood glucose meter (BGM) lactose measurement procedure are shown visually from left to right, and labeled for the corresponding step.

Phase 1: Extra data, example calculations and SAS code

1.) A 6.00% and a 12.00% lactose stock solution was prepared in DI water. The concentration of these stocks was later verified via the enzymatic spectrophotometric absorbance method (results in table A-2).

Table A-2 Lactose content of lactose stocks used in phase 1

Expected lactose content	Actual lactose content[1]	%CV[1]
6.00	6.02 ± 0.01	0.17
12.00	11.67 ± 0.04	0.36

[1] Measured via enzymatic spectrophotometric absorbance method. Reported as averages ± std. deviation. N=2

2.) The 6.00 and 12.00 % lactose stocks were used to prepare 0.1% and 0.2% lactose versions of the 6.94, 6.71, 6.35, and 6.05 pH PBS buffered stocks. The weights of the buffer, lactose stock, and enzyme used to make the dilutions were recorded for later calculations.

3.) The buffered stocks were all incubated with 2% lactase enzyme and measured using all of the BGM brands in table A-1. The pH of each measurement solution was tested again to ensure great shifts in pH did not occur to the buffer over the course of the experiment (see table A-3).

Table A-3 Post experiment pH of buffered lactose test solutions used in phase 1.

		Post experiment	
Buffer pH	Expected lactose content	pH	Temperature (°C)
6.94	0.2	6.89	22.5
6.94	0.4	6.88	22.5
6.71	0.2	6.67	22.5
6.71	0.4	6.63	22.5
6.35	0.2	6.32	22.5
6.35	0.4	6.32	22.5
6.05	0.2	6.03	22.5
6.05	0.4	6.05	22.5

4.) The theoretical glucose contents of each solution was calculated in Excel as follows (see figure A-4):

a. Recorded buffer, lactose stock, and enzyme weights were summed to calculate the total dilution weights (Column G=SUM (Columns C, E, & F)).

b. Grams of lactose in the dilutions were calculated (Column H = Column D * Column E/ 100).

c. Percent lactose in the dilutions were calculated (Column I= Column H/ Column G*100).

d. Theoretical percent glucose in the dilutions were calculated (Column J=Column I/2).

e. mg/dL glucose in the dilutions were calculated (Column K= Column J *1000).

A	B	C	D	E	F	G	H	I	J	K
Sample preparation (0.2000% lactose)										
pH of buffer	*Actual pH finished solution*	*g Buffer*	*Actual % lactose from spectro method*	*g ~6.0000% lactose solution*	*g Ezeco NL, lot 531901*	*g total solution*	*Theoretical grams lactose*	*Theoretical % lactose*	*Theoretical % glucose*	*Theoretical mg/dL glucose*
6.94	6.89	10.0059	6.0243	0.3596	0.1977	10.5632	0.021663265	0.2051	0.1025	102.5412
6.71	6.67	10.0042	6.0243	0.3534	0.1684	10.5260	0.02128976	0.2023	0.1011	101.1294
6.35	6.32	10.0087	6.0243	0.3610	0.1914	10.5611	0.021747604	0.2059	0.1030	102.9609
6.05	6.03	10.0061	6.0243	0.3589	0.2087	10.5737	0.021621095	0.2045	0.1022	102.2400
Sample preparation (0.4000% lactose)										
pH of buffer		*g Buffer*	*Actual % lactose from spectro method*	*g ~12.0000% lactose solution*	*g Ezeco NL, lot 531901*	*g total solution*	*Theoretical grams lactose*	*Theoretical % lactose*	*Theoretical % glucose*	*Theoretical mg/dL glucose*
6.94	6.88	10.0060	11.6704	0.3615	0.1872	10.5547	0.04218866	0.3997	0.1999	199.8572
6.71	6.63	10.0055	11.6704	0.3612	0.2206	10.5873	0.042153649	0.3982	0.1991	199.0765
6.35	6.32	10.0089	11.6704	0.3605	0.2017	10.5711	0.042071956	0.3980	0.1990	198.9952
6.05	6.05	10.0031	11.6704	0.3614	0.2244	10.5889	0.04217699	0.3983	0.1992	199.1566

Figure A-4 Theoretical solution glucose contents calculated in Phase 1.

5.) The percent bias difference (D) used to compare the BGM brands at each pH and lactose level were calculated in excel as follows (see figure A-5):

a. The difference between the meter output and the calculated dilution glucose content calculated in step 4 was computed for each solution result (Column F= Column D-Column E).

b. Percent bias difference was calculated using each difference result (Column G= Column F/ Column E *100).

	A	B	C	D	E	F	G
54	Meter readings (0.2% lactose)						
55	*Meter type*	*Theoretical % glucose*	*pH (Buffer used)*	*Meter response (mgldL glucose)*	*mg/Dl Glucose (measured)*	*mg/DL difference*	*%Bias*
56	ReliP	0.10	6.94	153	102.5412	50.4588	49.2083
57	ReliP	0.10	6.94	154	102.5412	51.4588	50.1835
58	ReliP	0.10	6.94	149	102.5412	46.4588	45.3074
59	ReliP	0.10	6.71	145	101.1294	43.8706	43.3807
60	ReliP	0.10	6.71	145	101.1294	43.8706	43.3807
61	ReliP	0.10	6.71	145	101.1294	43.8706	43.3807
62	ReliP	0.10	6.35	134	102.9609	31.0391	30.1465
63	ReliP	0.10	6.35	132	102.9609	29.0391	28.2040
64	ReliP	0.10	6.35	129	102.9609	26.0391	25.2903
65	ReliP	0.10	6.05	78	102.2400	-24.2400	-23.7089
66	ReliP	0.10	6.05	81	102.2400	-21.2400	-20.7746
67	ReliP	0.10	6.05	82	102.2400	-20.2400	-19.7965

Figure A-5 Example of how percent bias difference (D) was calculated. Example shows numbers for the for the ReliOn Prime meter brand at the 0.2% lactose experimental level, pH 6.05-6.94.

6.) SAS was used to perform 2-way ANOVA using pH and glucose level as class variables and percent bias difference (D) as the modeled parameter (figure A-6).

```
  title' pH impact by bias_Relip';
 data pH_vs_meter_bias_Relip;
infile datalines dlm='09'x;
input meter $ pct_glucose_target $ pH $ meter_bias;
datalines;
ReliP   0.10    6.94    49.2083153
ReliP   0.10    6.94    50.18353305
ReliP   0.10    6.94    45.30744431
ReliP   0.10    6.71    43.38066792
ReliP   0.10    6.71    43.38066792
ReliP   0.10    6.71    43.38066792
ReliP   0.10    6.35    30.14651034
ReliP   0.10    6.35    28.20402511
ReliP   0.10    6.35    25.29029726
ReliP   0.10    6.05    -23.70889503
ReliP   0.10    6.05    -20.77462176
ReliP   0.10    6.05    -19.79653067
ReliP   0.20    6.94    85.13216422
ReliP   0.20    6.94    89.13502182
ReliP   0.20    6.94    93.13787942
ReliP   0.20    6.71    89.87677184
ReliP   0.20    6.71    92.89068885
ReliP   0.20    6.71    92.89068885
ReliP   0.20    6.35    82.91901653
ReliP   0.20    6.35    66.3356991
ReliP   0.20    6.35    74.8786202
ReliP   0.20    6.05    18.49970348
ReliP   0.20    6.05    15.48699915
ReliP   0.20    6.05    11.97217744
;

proc print data=ph_vs_meter_bias_relip;
var meter pct_glucose_target pH  meter_bias;
run;

proc sort data=ph_vs_meter_bias_relip;
 by meter pct_glucose_target pH;
 run;

* proc glm procedure used to perform 2 way ANOVA for each meter's results separately,
Glucose level and pH are the class variables, meter bias is dependent variable;
proc glm data=ph_vs_meter_bias_relip plots=diagnostics;
 class pct_glucose_target pH;
 model meter_bias = pct_glucose_target|pH /ss3;
 means  pct_glucose_target pH pct_glucose_target*pH ;
 lsmeans pct_glucose_target pH pct_glucose_target*pH / stderr cl pdiff adjust= tukey lines;
 run;
```

Figure A-6 Example SAS code showing the statistical analysis performed in phase 1. The code shown is for the ReliOn Prime meter (a separate analysis was done for each meter).

Phase 2: Extra data, example calculations and SAS code

1.) To create a constant level of analyte measurable by the meters in the presence of different amounts of dairy ingredient, the lactose hydrolysis step was omitted in phase 2 and samples instead were spiked with glucose that could be measured directly. A common ~6.00% glucose stock was prepared for this purpose as per figure A-7:

   a. The grams of DI water and glucose monohydrate used to make the stock was recorded and summed (K26 = I26+J26).

   b. Percent glucose in the stock was calculated by factoring in the moisture content of the glucose monohydrate used (L26= (I26*0.90926)/K26*100).

	I	J	K	L
25	g glu-hydrate added	g DI	Total	% glucose in stock
26	6.5872	93.40	99.9872	5.99

Figure A-7 Calculation of glucose content in glucose stock used for phase 2.

2.) The glucose content of each finished ingredient model dilution was calculated as per figure A-8:

   The buffer, glucose stock, and DI water weights were summed to calculate total dilution weight (Column F=SUM (Columns C, D, & E)).

   a. Grams of glucose in each dilution was calculated using the known glucose stock concentration, which was 5.99% as per step 1(Column G = Column D * 5.99/ 100).

   b. Percent glucose in each dilution was calculated (Column H= Column G/ Column F*100).

   c. mg/dL glucose in each dilution was calculated (Column I= Column H *1000).

	A	B	C	D	E	F	G	H	I
41	solids type	% solids in buffer	g Respective solids solution	g 5.99% glucose solution	g DI water	g total solution	Theoretical grams glucose	Theoretical % glucose	Theoretical mg/DL glucose
42	N/a	0.00	10.0021	0.3594	0.2054	10.5669	0.0215	0.2037	203.7
43	WPI	2.00	10.0013	0.3554	0.1915	10.5482	0.0213	0.2018	201.8
44	WPI	4.00	10.0008	0.3560	0.1956	10.5524	0.0213	0.2021	202.1
45	WPI	6.00	9.9992	0.3558	0.1950	10.5500	0.0213	0.2020	202.0
46	WPI	8.00	9.9994	0.3540	0.1925	10.5459	0.0212	0.2011	201.1
47	MPI	2.00	9.9999	0.3551	0.1966	10.5516	0.0213	0.2016	201.6
48	MPI	4.00	10.0004	0.3569	0.1940	10.5513	0.0214	0.2026	202.6
49	MPI	6.00	10.0007	0.3583	0.1941	10.5531	0.0215	0.2034	203.4
50	MPI	8.00	10.0006	0.3524	0.1929	10.5459	0.0211	0.2002	200.2

Figure A-8 Theoretical solution glucose contents calculated in Phase 2.

3.) Percent bias difference (D) between the theoretical solution glucose contents and corresponding BGM outputs were calculated in a similar way as in phase 1 for each solution.

4.) SAS was used to perform 2-way ANOVA using added solids and solids type as class variables with percent bias difference (D) as the modeled parameter (Figure A-9).

```
 title "ReliP analysis";
data WPI_solids_vs_meter;
infile datalines dlm='09'x;
input Meter $ commodity $ PCT_solids $  PCT_Bias ;
datalines;
ReliP   WPI 0.000   118.9061404
ReliP   WPI 0.000   102.7090493
ReliP   WPI 0.000   111.5438263
ReliP   WPI 2.000   102.1504236
ReliP   WPI 2.000   95.70935619
ReliP   WPI 2.000   95.21388947
ReliP   WPI 4.000   101.3952471
ReliP   WPI 4.000   92.48833195
ReliP   WPI 4.000   95.95213227
ReliP   WPI 6.000   89.58276382
ReliP   WPI 6.000   88.5927755
ReliP   WPI 6.000   85.62281053
ReliP   WPI 8.000   82.01829113
ReliP   WPI 8.000   88.48342169
ReliP   WPI 8.000   85.00219754
ReliP   MPI 0.000   118.9061404
ReliP   MPI 0.000   102.7090493
ReliP   MPI 0.000   111.5438263
ReliP   MPI 2.000   87.50506653
ReliP   MPI 2.000   93.95365348
ReliP   MPI 2.000   101.3943307
ReliP   MPI 4.000   100.3729155
ReliP   MPI 4.000   101.3599742
ReliP   MPI 4.000   100.8664448
ReliP   MPI 6.000   92.74045185
ReliP   MPI 6.000   92.74045185
ReliP   MPI 6.000   95.19887598
ReliP   MPI 8.000   87.84046494
ReliP   MPI 8.000   91.83707057
ReliP   MPI 8.000   91.33749487
;
proc print data=wpi_solids_vs_meter;
var Meter commodity PCT_solids PCT_Bias;
run;

proc sort data=wpi_solids_vs_meter;;
 by Meter commodity PCT_solids PCT_Bias;
 run;


proc glm data=wpi_solids_vs_meter plots=diagnostics;
 class commodity PCT_solids;
 model PCT_Bias = commodity|PCT_solids /ss3;
 means commodity PCT_Solids commodity*PCT_solids;
 lsmeans commodity PCT_Solids commodity*PCT_solids / stderr cl pdiff adjust= tukey lines;

 run;
```

Figure A-9 Example SAS code showing the statistical analysis performed in phase 2. The code shown is for the ReliOn Prime meter (a separate analysis was done for each meter).

5.) The total solids of each solution was measured post experiment (Table A-4).

Table A-4 Total and corrected solids content of whey- and skim milk derived (WD and SMD respectively) model dilutions measured in phase 2.

		Post experiment measurements		
Solids type	Added solids target (%)	Total solids (%) [1]	CV (%)	Corrected added solids (%) [3]
NONE	0	1.06 ±0.05 [2]	4.69	0.00 ±0.07
WD	2	2.78 ±0.13	4.58	1.73 ±0.14
SMD	2	2.82 ±0.03	1.00	1.77 ±0.06
WD	4	4.59 ±0.18	3.86	3.53 ±0.18
SMD	4	5.11 ±0.04	0.69	4.05 ±0.06
WD	6	6.63 ±0.06	0.85	5.58 ±0.08
SMD	6	6.92 ±0.11	1.63	5.87 ±0.12
WD	8	8.58 ±0.04	0.41	7.52 ±0.06
SMD	8	8.85 ±0.04	0.40	7.79 ±0.06

[1] Reported as average ± S.D. N=2.

[2] Solids in the "none" solids type are derived wholly from buffer and spiked glucose.

[3] Calculated by subtracting the total solids of the "none" solids type category from each other category. Error propagated as $\sigma_{corrected\ solids} =$

$$\sqrt{(\sigma_{total\ solids,\ \text{'none' solids type}})^2 + (\sigma_{total\ solids,\ \ added\ WD\ or\ SMD\ solids})^2}$$

6.) The pH of each dilution was checked pre-experiment (Table A-5).

Table A-5 Pre-experiment pH of model whey and skim milk derived (WD -and SMD respectively) dilutions measured in phase 2.

		Post experiment pH measurement	
Solids type	Added solids target (%)	pH	Temperature
NONE	0	7.02	16.7
WD	2	7.01	18.5
SMD	2	6.92	18.1
WD	4	6.97	18.0
SMD	4	6.87	18.2
WD	6	6.94	18.3
SMD	6	6.80	18.5
WD	8	6.92	18.9
SMD	8	6.73	19.6

Appendix B - Collecting and analyzing data for phases 3 & 4

Phase 3 – Dairy ingredient dilution partitioning, extra data, and SAS code

1.) In light of phase 2 results, it was understood that material-specific dilutions would be required for an accurate BGM-based lactose analysis. This required sorting common dairy ingredients into categories based on approximate lactose contents that accommodated this need while also respecting the 20-500 mg/dL glucose range measurable by most blood glucose meters. See table B-1 for the justification behind the 0.2857, 0.0327, and 0.0053 target dilution factors specified in the work (corresponding to ~27, ~3, and ~0.5 % total "added solids").

Table B-1 Suggested dilution groupings for lactose analysis of dairy ingredients, arranged by increasing dry basis lactose content.

Example ingredient	Estimated lactose (% dry basis) [1]	Suggested dilution factor	Estimated post hydrolysis glucose in dilution (mg/dL) [2]	Estimated solids in dilution from dairy ingredient (%) [3]
WPI	0.20	0.2857	30	26.9
WPI	2.00	0.2857	280	26.9
MPI 85	3.50	0.0327	60	3.08
WPC 80	9.00	0.0327	150	3.08
MPC 70	18.00	0.0327	300	3.08
WPC 55	31.00	0.0053	80	0.50
Nonfat dry milk	51.00	0.0053	130	0.50
Sweet Whey	73.00	0.0053	190	0.50
Permeate	84.00	0.0053	220	0.50
Lactose	100.00	0.0053	260	0.50

[1] Adapted from a combination of industry specifications and Wisconsin Center for Dairy research, Dried Dairy Ingredients, 2nd Ed, January 1st , 2017.
[2] Calculated by dividing estimated diluted lactose percentage by 2 and multiplying by 1000.
[3] Includes initial dilution, lactase addition, and assumed 4% moisture content of dried dairy ingredients.

2.) For Phase 3, model ingredient dilution standards were prepared to simulate the above suggested dilution factors for both whey and skim-milk derived ingredients. Each

shared buffer + ingredient dispersion model dilution was partitioned into two sets of samples (calibration and verification series) that were spiked with lactose to create the specified range of lactose contents (0.08-0.62% for calibration, 0.12-0.58% for verification). The pH of these dilutions was measured after preparation, but before lactose was added (see table B-2).

Table B-2 Pre-experiment pH of model whey – and skim milk derived (WD- and SMD respectively) ingredient dispersions used to create the calibration and verification subsets of model dilutions measured in phase 3.

		Pre-experiment pH measurement	
Solids type	Target dilution factor	pH	Temperature (°C)
WD	0.0053	6.90	10.8
SMD	0.0053	7.02	22.3
WD	0.0327	6.93	14.5
SMD	0.0327	6.73	22.7
WD	0.2857	6.73	10.6

3.) The calibration solutions were measured using the BGM method and EZA reference method. The output from the meter was paired with the EZA lactose results to obtain linear calibration parameters (Table B-3); these parameters were calculated in SAS as exampled in Figure B-1.

Table B-3 Material and test strip lot-specific calibration parameters used in phase 3 & 4 for the Nova Max Plus and FreeStyle Precision Neo blood glucose meters. Note the non-comparable slope values for the strip lots tested in 27% whey derived (WD) model dilution, indicating strip lot specific calibration parameters should conservatively be used.

		Nova Max Plus				FreeStyle Precision Neo			
Model	Test strip lot #	Slope[†]	Intercept[††]	r^2	LOD (% lactose) [†††]	Slope[†]	Intercept[††]	r^2	LOD (% lactose) [†††]
0.5% WD	1	0.402 ±0.015	12.0 ±6.21	0.997	0.032	0.665 ±0.045	-5.12 ±17.8	0.991	0.055
	2	0.422 ±0.023	7.98 ±9.05	0.994	0.044	0.637 ±0.047	5.81 ± 18.6	0.989	0.060
	3	0.439 ±0.030	1.17 ±10.4	0.993	0.042	0.668 ±0.048	-0.928 ±18.8	0.990	0.058
3.0% WD	1	0.421 ±0.031	12.7 ±12.1	0.989	0.059	0.645 ±0.046	-4.81 ±18.2	0.990	0.058
	2	0.428 ±0.014	7.33 ±5.66	0.998	0.027	0.593 ±0.039	9.11 ±15.1	0.991	0.052
	3	0.445 ±0.034	2.96 ± 13.1	0.989	0.060	0.662 ±0.052	-0.933 ±20.3	0.988	0.063
27.0% WD	1	0.537 ±0.028	-3.69 ±7.55	0.995	0.031	0.707 ±0.036	-9.47 ±9.98	0.995	0.031
	2	0.544 ±0.021	-5.08 ±5.92	0.997	0.024	0.587 ±0.025	-11.0 ±6.70	0.997	0.025
	3	0.544 ±0.031	-5.69 ±11.9	0.990	0.041	0.547 ±0.041	-0.803 ±11.1	0.989	0.044
0.5% SMD	1	0.385 ±0.015	11.0 ±5.72	0.997	0.029	0.624 ±0.023	-5.26 ± 8.66	0.997	0.027
	2	0.390 ±0.018	9.94 ±6.75	0.996	0.034	0.563 ±0.047	10.1 ±17.7	0.986	0.062
	3	0.401 ±0.027	7.67 ±10.8	0.990	0.053	0.631 ±0.048	-3.01 ±18.2	0.989	0.057
3.0% SMD	1	0.449 ±0.017	-1.47 ±6.38	0.997	0.028	0.615 ±0.048	-8.12 ±18.1	0.988	0.059
	2	0.451 ±0.015	1.77 ±5.74	0.998	0.025	0.585 ±0.037	-3.04 ±13.8	0.992	0.047
	3	0.457 ±0.014	-0.527 ±5.39	0.998	0.024	0.621 ±0.051	-2.22 ±19.3	0.987	0.062

[†] Results expressed as parameter ±95% confidence interval. Units in (mg /dL glucose)/(mg/dL lactose)

[††] Results expressed as parameter ±95% confidence interval. Units in mg/dL glucose.

[†††] $\text{LOD} = \frac{3 \times RMSE}{slope}$, where RMSE is the root mean square error from linear regression model

```
title '0.5% MPI lot-specific Calibration, NMP';
Data ptfivepctmpicalibrationNMP;
infile datalines dlm='09'x;
input Expected meterlot1 meterlot2 meterlot3;
Datalines;
98.91   50  51  42
98.91   48  47  47
200.1   88  88  87
200.1   88  88  88
302.5   134 128 135
302.5   121 121 129
402.3   164 175 172
402.3   169 170 170
501.1   205 202 213
501.1   203 202 222
555.3   229 223 225
555.3   220 232 216
;

Proc print data= ptfivepctmpicalibrationNMP;
Var Expected meterlot1 meterlot2 meterlot3;
run;

Proc Means Data = ptfivepctmpicalibrationNMP N SUM MEAN STD MIN MAX;
Var Expected meterlot1 meterlot2 meterlot3;
run;

*Plot data first;
PROC SGplot Data = ptfivepctmpicalibrationNMP;
Scatter x=expected y=meterlot1;
Scatter x=expected y=meterlot2;
Scatter x=expected y=meterlot3;
run;

*Simple linear regression done on each strip lot's data.  "CLB" calls up 95% confidence intervals on parameters.;
PROC REG DATA= ptfivepctmpicalibrationNMP PLOTS=Diagnostics;
Model meterlot1=expected / P CLM CLB CLI;
run;

PROC REG DATA= ptfivepctmpicalibrationNMP PLOTS=Diagnostics;
Model meterlot2=expected / P CLM CLB CLI;
run;

PROC REG DATA= ptfivepctmpicalibrationNMP PLOTS=Diagnostics;
Model meterlot3=expected / P CLM CLB CLI;
run;
```

Figure B-1 Example SAS code for computing linear regression parameters and statistics for each calibration curve that was developed. Parameters pertaining to each lot of test strips were calculated individually. This example is for the 0.5% MPI subset of data measured using the Nova Max Plus meter.

4.) An example of how verification sample glucose meter readings were converted into percent lactose using the established calibration parameters is shown in figure B-2. Examples of how bias benchmarks AD & d between the BGM method and reference EZA method were calculated in excel are also shown in figure B-2. Example SAS code for calculating paired method regression statistics between the BGM and EZA method verification dilution results are shown in figure B-3.

a. Convert meter outputs to percent lactose using corresponding linear calibration parameters (Column I=(Column C-I$32)/(I$30*1000)).
b. Calculate AD for each reading (column O= ABS ((column I-column B)/column B*100%)).
c. Calculate d for each reading (Column U=column I-column B).
d. Calculate average AD for all readings in a grouping (O150=AVERAGE (O135:O146)).
e. Calculate average d for all readings in a grouping (U150 = AVERAGE (U135 :U146)).

	B	C	I	O	U
129			***Calibration slope 0.5% MPI, NMP #1***		
130			0.3851		
131			***Calibration intercept 0.5% MPI, NMP #1***		
132			11.0303		
133					
134	**% lactose (EZ reference result)**	**NMP #1 readings, mg/ dL**	**NMP #1 conversion (% Lactose)**	**NMP #1 absolute % diff (AD)**	**NMP #1 difference (d)**
135	0.1183	57	0.119	0.94	0.001
136	0.1183	56	0.117	1.25	-0.001
137	0.1975	85	0.192	2.73	-0.005
138	0.1975	85	0.192	2.73	-0.005
139	0.2950	134	0.319	8.23	0.024
140	0.2950	125	0.296	0.31	0.001
141	0.3846	159	0.384	0.09	0.000
142	0.3846	159	0.384	0.09	0.000
143	0.4717	186	0.454	3.68	-0.017
144	0.4717	192	0.470	0.37	-0.002
145	0.5541	223	0.550	0.66	-0.004
146	0.5541	235	0.582	4.97	0.028
147					
148				**NMP #1**	**NMP #1**
149				**Average absolute % diff (AD)**	**Average Difference (d)**
150				2.17	0.00
151				**Min AD**	**Min d**
152				0.09	-0.02
153				**Max AD**	**Max d**
154				8.23	0.03

Figure B-2 Example Excel spreadsheet detaining how glucose meter readings are converted into percent lactose based on calibration parameters, as well as how bias difference (d) and absolute percent bias difference (AD) relative to a control method are calculated. Note, only one-meter lot is shown (others are in hidden columns).

```
title '0.5% MPI lot-specific Verification, NMP';
Data threepctmpiverNMP;
infile datalines dlm='09'x;
input Expected meterlot1 meterlot2 meterlot3;
Datalines;
0.1183  0.119   0.123   0.130
0.1183  0.117   0.113   0.125
0.1975  0.192   0.192   0.193
0.1975  0.192   0.195   0.200
0.2950  0.319   0.315   0.320
0.2950  0.296   0.310   0.310
0.3846  0.384   0.382   0.402
0.3846  0.384   0.390   0.375
0.4717  0.454   0.467   0.472
0.4717  0.470   0.495   0.474
0.5541  0.550   0.554   0.551
0.5541  0.582   0.569   0.566
;

Proc print data= threepctmpiverNMP;
Var Expected meterlot1 meterlot2 meterlot3;

Proc Means Data = threepctmpiverNMP N SUM MEAN STD MIN MAX;
Var Expected meterlot1 meterlot2 meterlot3;

PROC SGplot Data = threepctmpiverNMP;
Scatter x=expected y=meterlot1;
Scatter x=expected y=meterlot2;
Scatter x=expected y=meterlot3;

*PROC REG procedure used to create linear slope & intercept parameters separately for each lot of test strips.
CLM calls up 95% confidence intervals for slopes & interceps, which should be statistically comparable to 1 & 0 for paired
method statistics ;

PROC REG DATA= threepctmpiverNMP PLOTS=Diagnostics;
Model meterlot1=expected / P CLM CLB CLI;
run;

PROC REG DATA= threepctmpiverNMP PLOTS=Diagnostics;
Model meterlot2=expected / P CLM CLB CLI;
run;

PROC REG DATA= threepctmpiverNMP PLOTS=Diagnostics;
Model meterlot3=expected / P CLM CLB CLI;
run;
```

Figure B-3 Example SAS code for calculating paired method regression statistics between the blood glucose meter (BGM) method and enzymatic spectrophotometric absorbance reference method results are shown. This example is for the 0.5% MPI subset of data, measured using the Nova Max plus

Phase 4 – Commercial dairy ingredient measurement extra data

1.) Commercial samples were diluted based on expected lactose concentration as specified in chapter 4 (see phase 3). The pH of each dilution was measured before the lactose concentration was measured via the BGM method (table B-4). Note, slight pH adjustments were needed for two of the higher solids samples, since the higher solids incorporation dictates that the native material pH, not the dilution buffer pH, characterizes the pH of the finished dilution.

Table B-4 The target dilution factors and dilution pH measured for each of the diluted commercial dairy ingredient samples for which lactose was measured in phase 4. The WPI #1 and #4 samples needed to have their pH adjusted using up using NaOH.

		Dilution pH		Adjusted dilution pH	
Ingredient	Target dilution factor	pH	Temperature (°C)	pH	Temperature (°C)
Milk Permeate	0.0053	6.80	6.8	-	-
NFDM LH	0.0053	6.92	6.7	-	-
NFDM HH	0.0053	6.98	4.9	-	-
Buttermilk	0.0053	6.73	6.9	-	-
MPC-70	0.0327	6.82	5.3	-	-
MPI-85	0.0327	6.84	5.2	-	-
Micellar Casein	0.0327	6.73	7.3	-	-
Whey Permeate	0.0053	6.71	6.6	-	-
Whey	0.0053	6.87	4.7	-	-
WPC 34	0.0053	6.78	6.1	-	-
WPC 80	0.0327	6.54	6.5	-	-
WPI #1	0.2857	5.99	24.1	6.51†	24.3
WPI #2	0.2857	6.56	10.7	-	-
WPI #3	0.2857	6.62	19.5	-	-
WPI #4	0.2857	5.88	23.3	6.50†	23.6

† pH adjusted up using minimal ~10% NaOH solution.

Appendix C - Enzymatic absorbance reference method

1.) Enzymatic absorbance spectroscopy was used as the reference method for lactose in this work. The method principally works as follows:

   a. Lactose is hydrolyzed to D-glucose & D-galactose at pH=6.6 by β-galactosidase.
   b. D-galactose is oxidized at pH=8.6 by nicotinamide-adenine dinucleotide (NAD+) to D-galactonic acid with the aid of β-galactose dehydrogenase. NADH is formed here.
   c. The amount of NADH formed in the reaction is measured via the absorbance of 340 nm UV-light, and is proportional to lactose concentration in the sample.

Figure C-1 Equipment and materials used for enzymatic spectrophotometric absorbance reference testing: A) Sample holder and chamber. B) Beckman-Coulter DU- 650 UV-VIS spectrometer. C) Lactose/D-Galactose reagent kit.

2.) Test kits containing all necessary reagents and general instructions were employed (Lactose/D-Galactose UV method, Cat. No. 10 176 303 035, Lot 305 78600, R-BIOPHARM), but calculations were carried out as recommended by Lynch and Barbano, 2007:

   a. Dilute samples as close to 0.05% as possible, recording sample weight (W_m) and added DI water weight in order to calculate finished dilution weight (W_p).

b. Filter samples as described in chapter 4.
c. Weight an empty 1 cm x 1cm disposable acrylic cuvette (W_c).
d. Add diluted, filtered sample to cuvette and record weight again (W_i).
e. Pipette necessary kit reagents directly into cuvettes as per kit instructions.
f. Place cuvettes in spectrometer, record background absorbance at 340 nm, remove cuvettes to add last reagent, and place back in spectrometer to allow the reaction to take place.
g. Record final, complete cuvette weight (W_t).
h. Calculate % lactose using data similar to C-1 using equations C-1, C-2, C-3, and C-4.

Equation C-1 Calculate weight of filtered sample (W_f) added to a sample cuvette for use in equation C-4.

$$W_f = W_i - W_c$$

W_i = diluted filtered sample weight +cuvette weight

W_c = cuvette weight

Equation C-2 Calculate total weight of solutions (W_s) added to a sample cuvette for use in equation C-4.

$$W_s = W_t - W_c$$

W_t = diluted filtered sample weight +cuvette weight +all kit reagents weight

W_c = cuvette weight

Equation C-3 Calculate change in absorbance (ΔA) for a sample for use in Equation C-4.

$$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$$

A_1 = Initial absorbance of each cuvette.

A_2 = Average of final 7 absorbance readings

Equation C-4 Calculation of lactose in sample measured via enzymatic absorbance spectroscopy.

$$C_{stock} = \bullet \frac{(W_s/D)\times MW}{\varepsilon \times W_f \times d \times 10000} \times \Delta A \times \frac{W_p}{W_m}$$

C_{stock} = concentration (g/ 100g) of lactose in sample

D = Density of assay solution of 1.0049 g/mL (Lynch & Barbano, 2007)

MW = molecular weight of anhydrous lactose, 342.3g/mole.

d = cuvette light path (1.00 cm)

ε = extinction coefficient of NADH (6.3L/mmol/cm at 340nm)

W_p = diluted sample stock weight

W_m = weight of original sample used in diluted sample stock solution

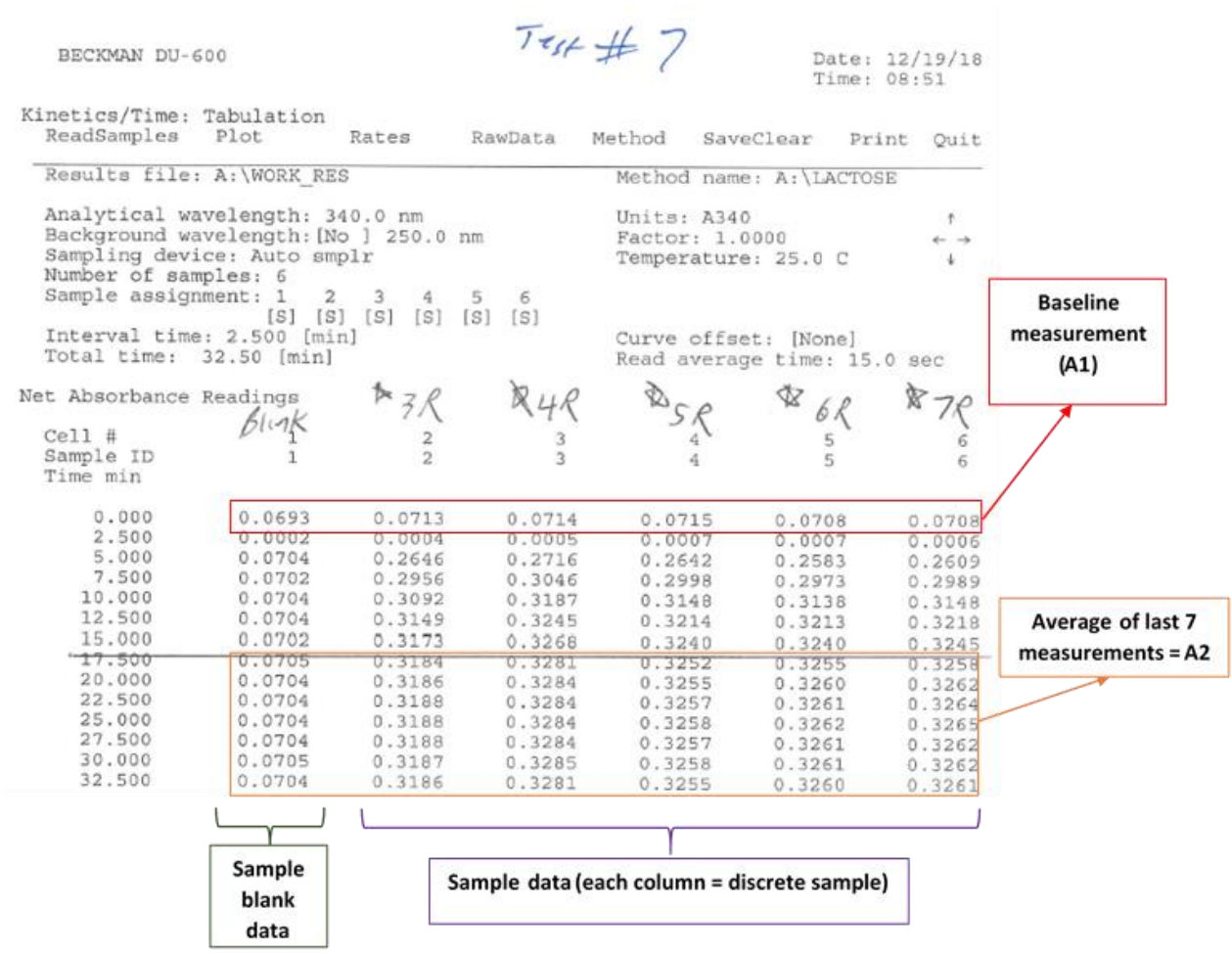


Figure C-2 Example absorbance spectrometer output for 5 test samples plus a blank sample reading. The columns identified as blank samples or test samples as well as what was used for absorbance readings A1 and A2 in equation C-3 are noted.

Appendix D - Alternative commercial biosensors

There are some proprietary commercial biosensors available in the marketplace to quantify lactose, including the Lactosens® (DirectSens GMBH, marketed by Chr. Hansen in the United States), BioMilk® ^{300} (Biolan, marketed by DSM), and YSI biochemistry analyzer (Xylem inc.). The author had the opportunity to trial the Lactosens® on a selection of the dried dairy ingredients. The Lactosens® gave acceptable lactose results in most of the samples tested (see figure D-1). Furthermore, the Lactosens® is third party validated and supposedly measures lactose directly (personal communication) and is therefore a good option when the direct measurement of small lactose amounts may be desirable (e.g. verification of lactose-free dairy products). As a comparison to the BGM method developed here, the Lactosens® is comparable, if not slightly more precise; however, the Lactosens® is a proprietary device, and the cost of consumables for the unit reflect this. From a general perspective, both biosensor-based methods (BGM and Lactosens®) were much less precise than the EZA method.

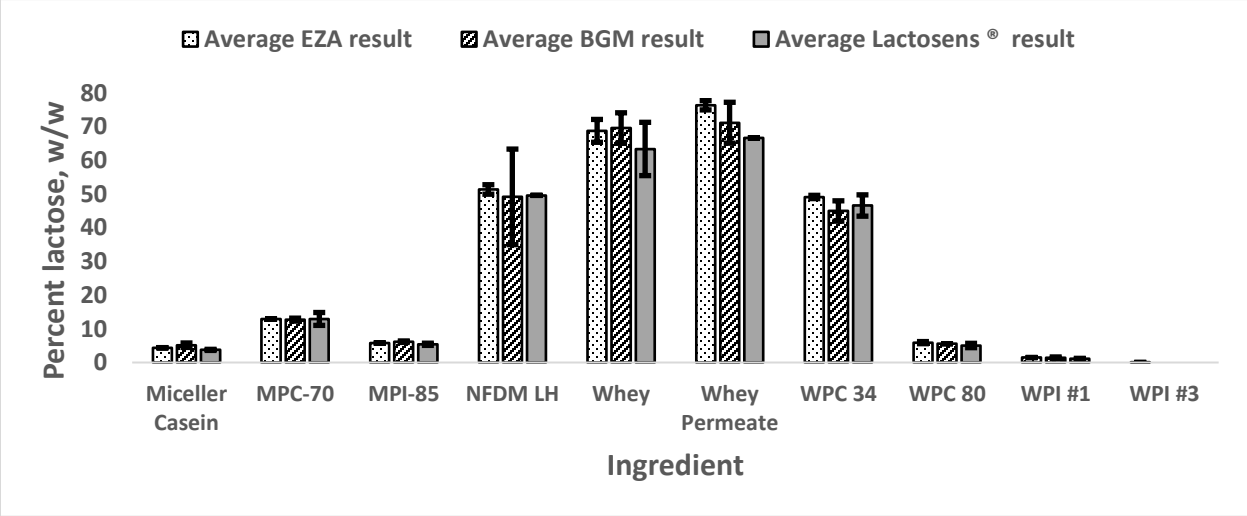


Figure D-1 – Lactose results for select commercial dried dairy ingredients, as measured by enzymatic spectrophotometric absorbance (EZA) method, blood glucose meter (BGM) biosensor method, and the Lactosens® proprietary lactose biosensor method. Bars represent average results; error bars represent 95% confidence intervals for each result (N=2). Note, only the EZA method could detect lactose in WPI #3.

Appendix E - Standard operating procedure for blood glucose meter- based lactose analysis in dairy ingredients

Method scope: Quantification of lactose in dried dairy ingredients that are between 0.2- 100% lactose as-is using blood glucose meter (BGM) biosensors.

1.) Use 1X PBS buffer adjusted to pH=7.0 for all dilutions to ensure a solution pH between 6.7 and 7 when possible.
2.) Prepare calibration standards that model the dairy ingredients to be ultimately measured.
    a. Target solution total solids of 0.5, 3.0, and 27% to represent 31-100%, 3.5-18, and <2.0% expected dry-basis lactose ingredients respectively. Non-lactose solids can be composed of WPI or Micellar casein to represent WD- and SMD ingredients respectively. Pure analytical grade lactose can be used to adjust the lactose content in the standards to between 0.08 and 0.6% lactose. Simply disperse the correct amounts of each powder and lactose into buffer using a consistent dispersion method.
3.) Create calibration curves by testing lactose in calibration standards using proposed BGM method and a reference method
    a. Combine 5.0g of diluted sample stock in a test tube with 0.1g of lactase enzyme (~245 yeast lactose units total), mixing thoroughly. Record the exact weight of diluted sample and enzyme used in order to calculate a dilution factor for this step (usually ~0.98).
    b. Incubate in a 40 °C water bath for no less than 15 minutes.
    c. Remove samples from the 40 °C water bath and immediately measure the created glucose in each dilution by dripping the solution onto the capillary site of a test strip corresponding to an optimal blood glucose meter (Nova Max Plus or similar performing).
    d. Develop linear test-strip lot specific calibration models by pairing the BGM results with results as measured by a trusted reference method (HPLC or EZA method recommended).

e. Freeze calibration solutions for later use with future test strip lots.

4.) Dilute real samples to a similar solids level as their corresponding calibration series
    a. Target dilution factors are 0.005, 0.0327, or 0.2857 to represent 31-100%, 3.5-18, and <2.0% expected dry-basis lactose ingredients respectively.
5.) Measure lactose in commercial samples using proposed BGM method (see steps 3A-C).
    a. Percent lactose in the original sample can be calculated using equation 4-1.