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Determination of Lactose by Reversed-Phase High Performance Liquid Chromatography.

Danessa Leann Sexton
East Tennessee State University

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Determination of Lactose by Reversed-phase High Performance Liquid Chromatography

A thesis presented to the faculty of the Department of Chemistry East Tennessee State University

In partial fulfillment of the requirements for the degree Master of Science in Chemistry

By Danessa Sexton
May 2004

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Mian Jiang
Tammy Davidson

Keywords: lactose, high performance liquid chromatography, reducing-sugars
ABSTRACT

Determination of Lactose by Reversed-phase High Performance Liquid Chromatography

By

Danessa Sexton

Lactose is the common disaccharide found in dairy products. It can, however, be a source of discomfort for those whom are lactose intolerant. Therefore, it is necessary to accurately, reliably, and efficiently determine the lactose content of foods. The goal of this research was to develop a reversed-phase HPLC method with UV detection for the determination of lactose. Aminobenzoic acid was used to prepare a derivative, making detection possible in the 300-360 nm region. The stationary phase is a C8 column, with 85% methanol 15% water mixture at pH 5 as the mobile phase. A linear response of peak area to lactose concentration of 0.018 mg/mL to 0.36 mg/mL was obtained with an estimated detection limit of 0.0036 mg/mL lactose concentration. The reproducibility was established with a 4.95% relative standard deviation. The average recovery was 107%, and results were in strong agreement with the standard alkaline ferricyanide method, establishing accuracy.
ACKNOWLEDGMENTS

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Carbohydrates are a diverse group of compounds with diverse functions. They serve as important dietary energy sources, structural and protective elements within cell walls, and lubrication for skeletal joints to name only a few. Carbohydrates are generally cyclic, polyhydroxy aldehydes or ketones. Many carbohydrates have the general empirical formula \((\text{CH}_2\text{O})_n\). Most carbohydrates are of the cyclic form, but may also have a straight chain form. In the straight chain form, if the carbonyl group is at the end of the carbon chain, it is an aldose. If the carbonyl is in the ketone form it is referred to as a ketose (1).

Carbohydrates are divided into three major classes: monosaccharides, oligosaccharides, and polysaccharides. Monosaccharides, often referred to as simple sugars, consist of a single aldehyde or ketone unit. The most abundant monosaccharide occurring in nature is glucose, which is derived from the simplest sugar, glyceraldehydes (2). Glucose is a six carbon sugar that is sometimes referred to as dextrose.

Oligosaccharides are composed of short chains of monosaccharide units joined by glycosidic bonds. Glycosidic bonds are formed when a hydroxyl group of one sugar reacts with the anomeric carbon (carbonyl carbon) of the other (1). The most common of the oligosaccharides are the disaccharides, made up of two monosaccharide units. Some of these disaccharides include lactose, comprised of glucose and galactose, and sucrose, comprised of fructose and glucose.
Polysaccharides contain 20 or more monosaccharide units. These long chains of monosaccharide units may be branched or linear. Cellulose, for example, is a linear chain, whereas glycogen has a branched chain. Some polysaccharides, such as starch and glycogen, serve as storage for monosaccharides used as fuel. Others, like cellulose and chitin, serve as structural and protective elements in plant cell walls, and exoskeletons (1). Polysaccharides are sometimes referred to as complex carbohydrates.

Most natural monosaccharides can be further identified by using their Fischer projection formulas. Fischer projections give a representative, three dimensional structure of the sugar on paper. Sugar molecules contain at least one asymmetric, chiral carbon, which makes them optically active. The two enantiomers are designated D and L. In the D form, the highest numbered chiral carbon has the hydroxyl group directed to the right. The L form is the opposite, with the hydroxyl directed to the left of the chiral carbon (2). In living systems, the D form of the sugars is the most common. The number of stereoisomers is related to the number of chiral centers, n. Generally, there will be \(2^n\) stereoisomers.

In general, monosaccharides with five or more carbon atoms occur mainly in the cyclic ring form. These ring structures are formed when the carbonyl functional group forms a covalent bond with the oxygen of a hydroxyl group. These derivatives are called hemiacetals or hemiketals and contain an additional asymmetric carbon that gives rise to two additional stereoisomers. The isomeric forms of these sugars that differ only in their configuration about the hemiacetal or hemiketal carbon are called anomers. These anomers are designated as either \(\alpha\) or \(\beta\) (1).
Monosaccharides can be termed reducing sugars if they are readily oxidized by mild oxidizing agents. In a reducing monosaccharide, the carbonyl carbon is oxidized to a carboxyl group. Disaccharides and polysaccharides may also be reducing sugars if they have a reducing end. In order to be a reducing sugar the disaccharide or polysaccharide must have a free anomeric carbon that is not involved in a glycosidic bond (1). Common reducing sugars include glucose, fructose, and lactose. Sucrose is not a reducing sugar.

**Lactose**

Carbohydrates are the most important global source of food energy. They represent 40-80% of the total food energy intake (3). The extent to which carbohydrates make up the diet is dependent upon the culture and location of the consumer. Carbohydrates are more economical than protein food sources for example and, therefore, comprise a larger portion of the diet of poorer and less developed countries. Common sources of food carbohydrates include grains, fruits, vegetables, sugar crops, root crops, and dairy products.

Lactose is one of these important energy sources and is sometimes referred to simply as milk sugar because it is the primary carbohydrate found in dairy products. It is the primary source of carbohydrates during mammal development. It constitutes 40% of energy consumed during the nursing period (1). Lactose has also been associated with the absorption of calcium.

The analysis of lactose is of great importance because it occurs in many types of foods. It is present in more than just milk and cheese where expected. Labeling terms can be used by the consumer to identify foods that contain lactose. Terms such as milk
solids, whey, curds, skim milk powder, and skim milk solids mean that lactose is present. Food groups containing lactose usually include breads and grains (e.g. muffins, biscuits, etc.), vegetables and fruits (e.g. instant potato products, vegetable dishes with cream or cheese sauces), milk and dairy products, meats (e.g. processed meats with lactose filler, breaded meats), and fats and snacks (e.g. creamy dressings, dessert mixes containing milk) (4).

Dietary polysaccharides are hydrolyzed to monosaccharides for metabolism. These polysaccharides are degraded by enzymes of the intestinal brush border, the microvilli of intestinal epithelial cells. Lactose is hydrolyzed to its two monosaccharide units, glucose and galactose, by the enzyme, lactase. Upon conversion, the monosaccharides are transported to the epithelial cells and are passed into the blood to be carried to various tissues to be phosphorylated and inserted into the glycolytic sequence (1).

Lactose intolerance occurs when there is a deficiency of lactase. With this deficiency, lactose is not completely digested and is converted by bacteria into toxic products within the large intestine (1). Symptoms of lactose intolerance include stomach cramps, bloating, flatulence, diarrhea, and nausea. The severity varies depending upon the degree of lactase deficiency.

Those whom are only mildly lactose intolerant may be able to tolerate lactose in small quantities. They may be able to drink milk in small servings of one cup or less (5). Hard cheeses also contain lower quantities of lactose which may be tolerated by the mildly lactase deficient. Cheddar cheese, for example, contains 4.5g of lactose per 100g sample compared to 52 g/100 g in milk (6).
Lactose intolerance is a prevalent disorder. An estimated 30-50 million Americans are diagnosed as lactose intolerant (5). It is even more prevalent in African Americans and Asians. Approximately 75% of African-Americans have low lactase levels (5). It is also reported that nearly 100% of Asians meet glucose-response or breath-hydrogen test criteria for lactose intolerance (6).

Lactase levels may decline later in life for some individuals. Human adult-onset lactase decline is characterized by the lactase enzyme activity during mid-childhood, and is reduced to <10% of childhood levels (7). The decline may begin between the ages of 5 and 7, but can occur as late as 20 (7). Adult-onset lactase decline is believed to be genetically inherited and is less prevalent in traditional milk-drinking societies of northwestern Europe (7). It is most prevalent in African and Asian populations.

Congenital lactose intolerance is rarer than adult-onset lactase decline. With congenital lactose intolerance, infants suffer similar symptoms to adults with lactose intolerance including flatulence, diarrhea, nausea, and bloating. The disorder is, however, more concerning when it occurs in infants since a large portion of their food energy is derived from the carbohydrates in milk. In order to cope with this problem, reduced- lactose and lactose-free infant formulas and products have been developed. Most lactose-free infant formulas are soy based and sweetened with sucrose. The sensitive, reliable analysis of lactose in infant products is of great importance to avoid discomfort and illness due to lactose maldigestion.
CHAPTER 2

TECHNIQUES FOR QUANTITATION OF LACTOSE

Lactose intolerance is a significant factor in choosing the diet of many sufferers. If lactose content is not monitored, it may cause severe discomfort and illness. Lactose occurs in a variety of foods other than milk and cheese. It is, therefore, important to reliably quantify lactose within these products. The chosen method should be one that is economical, rapid, and sensitive.

Enzymatic Methods

Enzymatic analysis is an old technique. During the 1880s analysis of carbohydrates, especially starch and sucrose, were performed using enzymes (8). Enzymes were not, however, widely used in the commercial laboratories until after World War II when spectrophotometers became more readily available in the analytical laboratory (8).

Enzymes are biological catalysts that are highly specialized proteins that catalyze reactions within biological systems. They typically have high specificity for their substrates, and they function under mild temperature and pH conditions (1). Most biological molecules tend to be quite stable and, therefore, react slowly under the mild conditions within the body. Enzymes provide an environment in which reactions are more energetically favorable. The substrate is bound to this active site of the enzyme and the reaction proceeds (1).

Enzymatic reactions are thought to be very specific, but the specificity depends upon the purity of the enzyme preparation (8). This becomes especially important when analyzing sugars that are very similar in structure and chemistry that may interfere with
One another. Other than specificity, enzymatic determination offers high sensitivity, rapid sample preparation for analysis, ease of automation, and no requirement for expensive equipment (8). The equipment used, however, must have good temperature control and be clean as basic requirements.

One disadvantage of enzyme usage is that some enzymes are quite expensive compared to other chemical reagents. They can also be degraded after prolonged periods at the temperature and reaction conditions required for the reaction to complete (8). Factors affecting enzymatic determinations must be carefully controlled. Of all the factors affecting enzyme activity, temperature is one of the most important. It is necessary to ensure that the temperature is as high as possible for the reaction to proceed as quickly as possible but also taking care not to denature the enzyme. Most successful analyses control the temperature to at least +/- 0.1 °C (8).

Most enzymes have a narrow pH range within which they have a maximum activity. Buffer solutions are usually necessary to maintain the substrate at the optimum pH and ionic strength (8). Enzymes exposed to pHs outside the optimum range may be denatured and rendered ineffective. Recommended pH ranges should be followed precisely to prolong the activity of the enzyme.

Enzymatic activity can be further altered by inhibitors and activators. Substrates that are used during the reaction, other than the intended substrate, reactive compounds containing heavy metals, and the products themselves may act as inhibitors (8). Inhibitors will slow the enzymatic reaction by binding preferentially with the enzyme or product thus preventing the intended substrate to bind to the enzyme. Activators, on the other hand, increase the speed of the reaction.
The measurements of the products of the enzymatic reactions are performed by various techniques. Spectrophotometry is one of these preferred methods. If the reaction products absorb in the UV, visible, or infrared regions the concentrations can be measured directly. A double-beam spectrophotometer is most commonly used where the measurements are carried out and referenced against a blank cuvette containing all reactants except the substrate (8). When the products do not absorb to an appreciable extent, indicators may be used to produce colored complexes which may then be measured (8).

Enzyme electrodes may also be used for analysis of carbohydrates. The enzyme can be immobilized upon a membrane or film. For carbohydrate analyses amperometric detection is most often used (8). Electrodes for the analysis of glucose, maltose, galactose, lactose, and sucrose have been reported. The measurement of glucose with glucose oxidase immobilized on the electrode is the most commercially feasible (8). Galactose oxidase electrodes for the analysis of galactose and lactose are not very specific, and the determination of maltose and sucrose is quite complicated, requiring more than one enzyme (8).

Recently, Antiochia and Palleschi developed a tri-enzyme electrode for the sequential determination of fructose and glucose within the same sample (9). The electrode had three enzymes, glucose dehydrogenase (GDH), fructose dehydrogenase (FDH), and diaphorase. FDH catalyzes the oxidation of fructose using a mediator as the electron acceptor followed by the oxidation of glucose by GDH coupled with the diaphorase enzyme and avoids the application of a high over potential for the reoxidation of the NADPH at the electrode surface (9). The probe is added to a solution containing...
the analytes of interest and an electrochemical mediator, and the current signal is due to
the fructose. NAD(P)\(^+\) is then added and a current response due to the glucose is
measured. The overall reaction scheme is described by three reactions (9).

\[
\text{FDH}
\]
\[
\text{Fructose} + \text{mediator}_{\text{ox}} \rightarrow \text{chetofructose} + \text{mediator}_{\text{red}} \quad (1)
\]

\[
\text{GDH}
\]
\[
\text{Glucose} + \text{NAD}^+ \rightarrow \text{gluconolactone} + \text{NADH} \quad (2)
\]

\[
\text{NADH} + \text{mediator}_{\text{ox}} \rightarrow \text{NAD}^+ + \text{mediator}_{\text{red}} \quad (3)
\]

The instrument consisted of a platinum working electrode, a silver-silver chloride
reference electrode, and an ABD amperometric biosensor detector. The optimum
conditions for analysis were pH 7.0 using phosphate buffer and a temperature of 30°C.
The linear dynamic range using standard fructose and glucose solutions was between 5 \(\times\) \(10^{-6}\) and 2 \(\times\) \(10^{-4}\) mol/L with a lower limit of detection of 1 \(\times\) \(10^{-7}\) mol/L (9). The relative
standard deviation was determined to be 3.5% illustrating good reproducibility of
measurements. The probe measurements were then compared to spectrophotometric
measurements of commercial honey samples containing both glucose and fructose. The
relative standard deviation using the probe was 3.3% compared to 4.4% using the
spectrophotometric method. The percent error in measurements of fructose and glucose
was found on average to be less than 10% (9).

Many methods have been studied for the development of enzymes on
electrochemical probes, but a method reported by Stoecker. pursues a method of
immobilizing galactose oxidase on a solid resin for the development of a galactose
oxidase column. Galactose oxidase is the enzyme that catalyzes the oxidation of galactose to produce an aldehyde at the sixth carbon (10). This reaction also produces hydrogen peroxide, which the authors detect by DC amperometry. However hydrogen peroxide can also be detected by chemiluminescent methods.

In this study, galactose oxidase was immobilized upon a NHS-activated HiTrap column. Solutions were buffered to pH 7.4 for the analysis using phosphate buffer. After reacting the galactose sample on the column, the solution was pumped to the amperometric detector where the peroxide was measured at a potential of 0.55V. The minimum detection limit was 50µM, which is not quite as favorable as some other methods, but the immobilized enzyme offers the benefit of greater enzyme stability (10).

Hamid, Moody, and Thomas also focused on the amperometric measurement of hydrogen peroxide in their multi-enzyme electrode design to measure lactose (11). This design amplified the signal output by using the concept of substrate recycling. Here, the products of the first enzymatic reaction are the substrates for the subsequent reactions. In this study, lactose was hydrolyzed with the galactosidase or lactase enzyme to produce glucose and galactose. Glucose and galactose were then oxidized with the glucose oxidase and galactose oxidase enzymes respectively. Both reactions produced hydrogen peroxide, which was monitored at the platinum electrode (11).

This tri-enzyme electrode was applied the determination of lactose in milk samples. The samples were pre-treated to remove protein, and the enzymes were immobilized on nylon net. Optimum pH for this analysis was established at 8.5. As is expected for an enzymatic reaction, high response was detected over a narrow pH range. The electrode was also studied for thermal stability. It was reproducible for temperatures
up to 42° C. The tri-enzyme electrode showed good sensitivity for the determination of lactose with a linear range of $3 \times 10^{-6}$ to $2 \times 10^{-3}$ M (11).

**Redox Methods**

Quantitation of sugars with reducing properties can be performed by titration with an oxidizing agent. This method is quite simple and inexpensive. No expensive or sophisticated equipment is required. It makes determination of reducing sugars in the presence of non-reducing sugars such as sucrose possible without interference. However, some biological compounds may interfere with the analysis, and temperature must be carefully controlled.

One such technique involves the titration of the sugar-containing solution with a cupric ion complex. The Benedict’s reagent containing a cupric citrate complex, and the Fehling’s solutions, containing a cupric tartrate complex, are two such oxidizing agents (12). In both cases, as the titrant oxidizes the aldose, a brick-red Cu$_2$O precipitate is formed. The titration is performed under alkaline conditions to convert ketoses to aldoses, which are in turn oxidized by the copper complex solution (12). The change is quite visible since the original copper complex solution has a vivid blue color.

For analysis of reducing sugars using the Fehling’s reagent several factors play an important role upon the accuracy of the technique and must be carefully controlled. Quimsburg and Thomas performed an early study to determine and control such factors that they believed to have a significant effect on the accuracy of the method including temperature, auto-reduction of the Fehling solution, and reducing action of sucrose on the Fehling reagent (13). The temperature of the Fehling reaction is often controlled using a simple water bath. Quisumbing and Thomas noted this as cause for concern because the
temperature is quite difficult to control with great precision. This could lead to variation in the completeness of the reduction and lead to irreproducible results. Within this study, a temperature range of 60-110°C was studied to determine the optimum temperature. It was determined that 80°C is appropriate for a reaction time of 30 minutes (13). Temperatures less than this require an hour or more to achieve the same results.

It was further noted that error may arise when sucrose is present within the sugar mixture. Munson and Walker proposed tables for the determination of invert sugar and lactose in the presence of sucrose (13). Quisumbing and Thomas worked to find the optimum conditions under which to eliminate or reduce to a minimum the reducing action of sucrose so as to eliminate its interference. It was determined that if the concentration of reducing sugar is high enough to precipitate the copper in the Fehling’s solution, sucrose played little or no part in the reduction (13).

Modifications to the methods using the Fehling’s reagent have been suggested and performed. One of these is the Lane-Enyon method (14). In this procedure, the reaction of the Fehling’s solution and the reducing sugar is followed in a two-stage titration. In the first stage, the bulk of the sugar solution is added, and the then the remaining volume, ≤ 1 mL, is added drop-wise. An internal redox indicator, such as methylene blue, is used to clarify the endpoint. Reference to standardized tables is necessary to determine the concentration of reducing-sugars. Different tables are required for each reducing sugar because under the alkaline conditions, each sugar is degraded differently (14).

Experimental conditions for the Lane-Enyon method must be carefully controlled to obtain accurate results. Heating time, temperature, and reactant concentrations will
affect the results. Sugar solutions must be nearly neutral (14). No other reducing agents should be present within the sample as these too would alter the results.

The Munson-Walker method also uses the Fehling’s reagent for quantitation of reducing sugars. In the Fehling method, a known, fixed volume of sugar solution is heated with excess Fehling’s solution. The resulting cuprous oxide may then be washed, dried, and weighed to determine the sugar content by gravimetric calculation or titration with potassium permanganate or sodium thiosulfate. For titration with potassium permanganate, the cuprous oxide precipitate is dissolved completely in acidic ferric sulphate, where the cuprous ions reduce the ferric ions to ferrous. The resulting ferrous ions are then titrated with the permanganate. To perform the titration with sodium thiosulfate, cuprous oxide is dissolved in nitric acid to be oxidized to cupric nitrate. Potassium iodide is then added resulting in the oxidation of iodide to iodine to be titrated with thiosulfate using a starch indicator (14).

Another quantitative method based upon the reducing character of a selected sugar is iodometric titration. Iodine preferentially oxidizes aldose sugars but not ketose sugars under alkaline conditions. With alkaline conditions, iodine is converted to hypoiodite and excess iodine is titrated with thiosulfate (14). This technique can be advantageous for the determination of glucose, lactose, and galactose when fructose is present. Fructose is a ketose, and therefore would contribute no interference in the determination of the other reducing-sugars.

**Polarimetry**

When a plane of polarized light is passed through a solution with a chiral, asymmetric component, the light is bent. It is refracted to the right or to the left
depending upon the structure of the chiral component. As previously mentioned, carbohydrates are chiral. The convention of naming the stereoisomers + or - is based upon the direction to which they rotate the plane of light compared to glyceraldehyde. The L- and D- designations refer to levorotary, rotating to the left, and dextrorotary, rotating to the right (1). Glyceraldehyde, the simplest sugar, is used as a reference for assigning the designation. Stereoisomers with structures similar to L-glyceraldehyde are designated L, and those similar to D-glyceraldehyde are designated as D (1).

\[
\text{CHO} \\
\text{HO} \\
\text{CH}_2\text{OH}
\]

L-Glyceraldehyde

\[
\text{CHO} \\
\text{H} \\
\text{OH} \\
\text{CH}_2\text{OH}
\]

D-Glyceraldehyde

To use this phenomenon for quantitation of sugar content, the specific rotation of light must be measured. The specific rotation, \( \alpha \), may vary with the concentration of solute within the analyte. In order to make an accurate prediction about the concentration, the solvent used must not rotate light. It must not have chiral components.

For the sugars, glucose, sucrose, and fructose, the specific rotations vary with concentration in an unrelated fashion, and sucrose’s specific rotation varies the least of all.
with changing concentrations. Mathematical treatment relates the specific rotation to concentration by the following equations at 20°C.

\[
\text{Sucrose } \alpha = +66.46 + 0.0087c - 0.000235c^2 \quad (4)
\]
\[
\text{Fructose } \alpha = -0.258c - 88.13 \quad (5)
\]
\[
\text{Glucose } \alpha = +52.50 + 0.0227c + 0.00022c^2 \quad (6)
\]

The specific rotation of lactose does not change with concentration (14).

As it can be observed, this technique requires a mathematical treatment of the measured specific rotation. It is not a suitable technique for quantifying lactose. Many other factors can influence the data collected by this technique. The rotation of the light is influenced by the wavelength of light used and the distance the light path must travel through the solution. Of these, the temperature is the most difficult to control, but it is necessary to control the temperature carefully in order for this technique to give a reasonable estimate of analyte concentration (14).

**High Performance Liquid Chromatography**

High performance liquid chromatography is a widely used technique for the analysis of complex mixtures. Because of this, it is especially widespread in food analysis. The reasons for the popularity of HPLC include sensitivity, separation ability of non-volatile compounds, and its widespread applicability to substances that are of strong interest to industry, science, and the public at large. Some of these substances of interest include, amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, terpenoids, pesticides, antibiotics, steroids, metal-organic species, and others too numerous to mention (15).
In all chromatographic techniques, the sample is transported or carried by a mobile phase. This mobile phase may be a gas, liquid, or supercritical fluid depending upon the chosen technique. With HPLC, as the name suggests, the mobile phase is a liquid. The mobile phase and sample are forced through a column containing the stationary phase.

The mobile phase and stationary phase are the soul of the chromatographic technique. Both must be carefully chosen to achieve separation of the components of the mixture of interest. The phases are chosen so that some components are retained longer on the stationary phase while others are transported out of the column more quickly. Species that have a strong affinity for the stationary phase are retained longer while those with a stronger affinity for the mobile phase are eluted more quickly. HPLC allows the analyst to take advantage of differences, even very slight differences, to separate components of mixtures. This is done by choosing an appropriate column and mobile phase combination.

Reversed phase HPLC consists of a nonpolar, usually hydrocarbon, stationary phase with a polar mobile phase. Examples of the stationary phase would be the use of a C8 or C18 column. This column consists of an eight or eighteen carbon hydrocarbon chain bonded onto a solid support, such as silica. The mobile phase would be polar solvents such as methanol, water, or a combination thereof. Reversed phase HPLC is the most popular liquid chromatography technique. It has wide applicability to a diverse group of compounds. Once the stationary phase is chosen, the mobile phase may be altered to increase or improve separation. One may vary the composition of the mobile
phase by making it more or less polar, adjusting pH, adding ionic salts, or adding surfactants.

A mobile phase consisting of purely water has been explored because of the desirable characteristics it may present. A 100% aqueous mobile phase offers the advantages of being a very inexpensive and non-toxic eluent. However, reproducibility of C18 and C8 stationary phases, the most common columns used for reversed-phase HPLC, decreases when the eluent is more than 98% aqueous. This problem has been explained as a collapsing of the stationary phase hydrocarbon chain when exposed to pure water. If the organic composition of the mobile phase was too low, the stationary phase would collapse onto itself in a low-energy conformation (16).

Verharr, Kuster, and Claessens further explored the application of aqueous mobile phases to reversed-phase stationary phases for the analysis of starch syrups. One of the variables examined was pH. The 100% water mobile phase was adjusted to pH values of 6.5 and 2.0 using 0.005 M sulfuric acid, and pH 10.0 using triethlyamine (17). It was noted that the low pH, 2.0, had no significant effect upon retention times or resolution. However, slightly improved resolution was observed with the higher pH, 10.0, but this slight improvement was determined not to be valuable enough to risk degradation of the column at high pH (17).

The addition of salts to the mobile phase was also explored. Chromatograms of the same syrup samples were ran with a mobile phases of 100% water, and 0.5 M sodium chloride, and 1.0 M sodium chloride were added to the samples. The salt-free sample resulting in a disturbed baseline, but those samples containing salt showed
improved resolution of the analyte peaks. It was also observed that the addition of small amounts of primary alcohols, methanol and pentanol, improved baseline noise (17).

Normal phase HPLC can be thought of as the opposite of reversed phase. With normal phase, the stationary phase is more polar while the mobile phase is more nonpolar. Normal phase is less popular than reversed phase HPLC. It makes up only about 25% of the HPLC techniques currently in practice. However, the polar functional group is quite applicable to certain compounds. Common functional groups of normal stationary phases include cyano, diol, amino, and dimethylamino functional groups (15).

The typical HPLC instrument consists of one or more solvent reservoirs, pump, an injector or auto-sampler, a column, and a detector. The setup and control of the various components can vary depending upon the sophistication of the equipment, but all HPLC instruments include those basic parts.

The mobile phase is contained in one or more solvent reservoirs connected to a high-pressure pump. The solvent must be thoroughly degassed by sparging with an inert gas such as helium. This may be done either using an automatic degasser attached to the instrument or performed separately. The mobile phase typically consists of an organic and aqueous phase where the proportion of each varies depending upon the stationary phase and analyte to be tested. The mobile phase may be pre-mixed in the appropriate ratios or mixed while pumping if the equipment is capable. Automatic mixing using the attached HPLC pump with a proportioning valve usually leads to a more homogeneous mixture and therefore a more accurate and reproducible chromatogram.

Both the pump and the injector are attached to the column. The pump consistently delivers mobile phase through the column to carry the analyte which is
introduced via the injector. The injector may be a single manual injector or an auto-
sampler capable of injecting a sequence of several samples automatically. The auto-
sampler, of course is preferred for a large number of samples.

As previously mentioned, the analyte is carried through the stationary phase of the
column by the mobile phase. The column contains the crucial stationary phase upon
which the chemical interactions occur that separate the components of the mixture. As
the components exit the column, they enter the detector. Many detectors are available
and used for HPLC analysis.

The type of analyte is determinate in the choice of detector for the HPLC
instrument. Some samples will elicit a response by some detectors, but give no signal
with others. The most widely used is the UV detector. This particular detection system
is problematic for the analysis of mono- and disaccharides such as sucrose, glucose,
fructose, and lactose. These sugars absorb in the 190-210 nm wavelength range (14). In
this region, unless the mobile phase and samples are completely purified or complete
resolution is achieved, interference will be encountered. Selectivity is very difficult to
achieve in this region.

UV detectors are one of two forms. The simplest UV detector is a filter
photometer with a mercury lamp as the radiation source. The 254 nm wavelength is most
commonly isolated by the filters, but some instruments can also use the 250, 313, 334,
and 365 nm wavelengths by substituting filters (15). Modern instruments are usually
equipped with filters that can be changed by computer control.

More sophisticated UV detectors are equipped with monochromators.
Instruments that allow scanning a wavelength range require monochromators. Some of
these are limited to the UV region for analysis while others can scan both the UV and visible regions for analysis. Wavelength selection is often computer controlled. The most powerful UV detector is the photodiode array. Manufacturers of the diode array offer instruments that permit the collection of data for an entire spectrum in approximately one second (15).

Carbohydrates only absorb to an appreciable extent in the 190-210 nm wavelength region. It is quite difficult to overcome interferences in this region from other organic species. However, Owens and Robinson used UV detection for the analysis of sheep plasma at 190 nm (18). The sample was carefully purified before analysis. Samples of blood and amniotic fluid were collected and deproteinised by the addition of zinc sulfate before being deionized by the passage through tandem cation and anion exchange columns followed by water washing. The hydrophobic compounds were removed by passing through a C18 column. The HPLC system used for analysis consisted of a pump for solvent delivery, a spectrophotometer set at 190 nm, and an integrator. The column was a Waters Sugar-Pak column, Ca$^{2+}$ cation exchange with water as the mobile phase. Recoveries of the carbohydrates present were reported as 94%. Overall, the method was more sensitive than detection with refractive index. The reported limits of detection for glucose, fructose, and galactose were 5.5, 1.6, and 3.7 nmol, respectively.

Fluorescence detectors are also sometimes employed for liquid chromatography. The emitted light intensity is collected and measured at a 90° angle compared to the excitation beam (19). This is done in order to reduce scattering. On average, fluorescence is more sensitive than UV. It has an approximate lower detection limit of
for strongly fluorescent compounds without interference, compared to $10^{-11}$ for UV detection. Once again, however, without derivitization, this method of detection is not applicable to most sugars because they lack a fluorophore.

Fluorescence detection offers high sensitivity and good selectivity and many techniques for the derivatization of carbohydrates for fluorescence detection have been proposed to attempt to take advantage of these benefits. Once the derivative is formed, it may also make the carbohydrate of interest detectable by UV detectors. This is advantageous because UV detectors are quite inexpensive and readily available for use in most analytical laboratories. However, by adding another step in the analytical process of making a derivative, the analyst must be careful not to introduce additional error and/or reduce reproducibility. The derivatization process must be quantitative, non-destructive, specific to species of interest, and by-products and/or excess reagents must be easily removed so as not to introduce additional interferences (20). Most derivatization methods are directed to the reducing end of the sugar molecule where key functional groups reside such as, carbonyl, hydroxyl, and free amino groups.

Reductive amination is a popular method of derivatization for the analysis of sugar molecules. Tagging of the reducing end with an aromatic compound having a primary amino group by reductive amination is the most popular method used for the analysis of oligosaccharides. Derivatives of this nature are stable against acidic and basic conditions, thus making a range of eluent pH’s possible (20).

Derivatives of p-aminobenzoic acid prepared through reductive amination have been developed to enable fluorescence detection $\lambda_{ex}= 313$ nm and $\lambda_{em}= 358$ nm or
spectrophotometric determination at 303 nm (21). The reaction proceeds by the mechanism below.

The lone pair of the amino group of p-aminobenzoic acid, PABA, attacks the reducing end of the sugar resulting in ring opening. A reducing agent, such as sodium cyanoborohydride, then breaks the double bond between the carbon and nitrogen resulting in the final product.

Derivatives of p-aminobenzoic acid of glucose, lactose, galactose, mannose, xylose, and arabinose have been prepared (21). The purpose of these derivatives was for the analysis of soil samples. Both C18 and anion exchange columns were employed, as well as, UV and fluorescence detection. The mobile phase consisted of a methanol water mixture with the pH adjusted over a wide range. Under these conditions, the author
reported minimal separation of glucose and galactose derivatives only at pH less than 3, and resolution of lactose was not achieved as it co-eluted with p-aminobenzoic acid. Detection limits for the sugar derivatives using both fluorescence and UV were 20-75 µg/L (21).

Refractive index detectors are another of the most widely used detectors for pairing with HPLC and are quite possibly the most common detectors for the analysis of sugars because no fluorophore or chromophore is necessary. This detector operates under the principle that the refractive index changes depending upon the refracting or light bending properties of liquids (19). Any solute present within the solvent elicits a response. There is a direct correlation between concentration and refractive index change. The detector operates by passing light through a cell with the eluent passing through and also through a cell with a reference. The difference in the light is then measured and reported as the detector response.

The refractive index detector, while applicable to sugar analysis, has some distinct disadvantages. Refractive index is a physical response that when altered by any component or condition will give a response. It is non-specific and any dissolved solute will give a signal. For food samples, this presents a strong disadvantage because the sample matrix contains many components that will all produce signals from the RI detector. Refractive index is also quite sensitive to changes in temperature, pressure, and solvent composition. All solvents for mobile phases must be carefully degassed, and gradients are not possible. The detector itself must be carefully thermostatted to avoid temperature fluctuation. The column must also be temperature controlled because the temperature of the column effects the equilibrium of the solvent particles in the mobile
phase and those of the stationary phase thus resulting in compositional changes of the eluting mobile phase (14).

The refractive index detector also suffers from low sensitivity when compared to other modern detection methods. Column loadings of approximately 40 micrograms are required for precise quantitation by modern RI detectors (14). This is not a large concern for sugar analysis within food samples because sugars are typically present as a major component. However refractive index is not a suitable choice for trace analysis.

Despite its disadvantages, the refractive index detector is commonly used for the analysis of sugar mixtures since no derivatization is required. A common instrumental set-up consists of a specialty carbohydrate column usually with an amino functional group, a mobile phase mixture of acetonitrile and water, and a refractive index detector. One such study aimed to separate mono- and disaccharides and sorbitol. Using a Waters carbohydrate column, a refractive index detector and a mobile phase of 80% acetonitrile, 15% water, and 15% ethanol separation of fructose, glucose, sucrose, sorbitol, maltose, and lactose was achieved. However, the analysis was quite slow requiring 30 minutes at a flow rate of 1.8 mL/min or 22 minutes with a flow rate of 2.2 mL/min (22).

A similar study by Rjakyla compared reversed-phase columns with varying hydrocarbon chain lengths, a pure water mobile phase, and refractive index detection (23). Hydrocarbon chain lengths varied between 6 and 18 carbons in the stationary phase. The column temperatures ranged 5-25°C, with water as the eluent at a flow rate of 1.0 ml/min. It was noted that decreased column temperature led to increased retention times and better resolution of the sugar peaks within corn syrup samples with the most suitable temperature being 15°C. It was further noted that, as expected, with less
hydrophobic stationary phases, the retention times decreased. Using these conditions separation of fructose, maltose, and sucrose was achieved.

Electrochemical detection has found much applicability to food analysis when coupled to HPLC. A typical amperometric detector consists of three electrodes, a reference, an auxiliary, and a working electrode. The eluent enters the detector and is passed through a channel over the working electrode to which a voltage is applied and the current is monitored with time. The current is amplified to provide the response (19).

Single potential instruments, while providing good selectivity and signal to noise ratios, are not appropriate for carbohydrate detection because electrochemical reaction products deposit on the electrode thereby poisoning it (14). This electrode poisoning problems can be avoided by using a pulsed amperometric detector. In a three potential cycle, the hydroxyl groups of the carbohydrate molecule can be oxidized at the first potential. The gold electrode surface is then oxidized to remove reaction products and finally reduced to its initial state by the application of the third voltage. With this instrument configuration, the current is only monitored during the first stage of oxidation. This method of detection is significantly more sensitive than refractive index. The on-column load for precise detection is 1-8 µg.

Electrochemical detection is often used in conjunction with enzymatic reactions. However, a recent study using anion exchange chromatography coupled with pulsed amperometric detection achieved separation of eleven sugars including, but not limited to, glucose, galactose, and fructose without prior derivatization. The analysis was applied to lake water and soil extracts. Carbohydrates were detected by measuring the electrical charge generated by their oxidation at the surface of a gold electrode. The sequence of
potentials was $E_1 = 0.05$ V, $E_2 = 0.75$V, and $E_3 = -1.15$V. Anion exchange columns with mobile phases containing NaOH were used. Detection limits were 0.02-0.05 µmol/L with recoveries of 40-83% with low recoveries observed primarily for fructose (24).

Pulsed amperometric detection was also applied to the determination of carbohydrates in milk and milk products (25). Raw milk samples from cow, sheep, buffalo, and goat were obtained and treated to remove fats. The detector was made of a gold working electrode and a Ag/AgCl reference electrode with potentials of $E_{\text{det}} = 0.05$, $E_{\text{ox}} = 0.80$, and $E_{\text{red}} = -0.22$ V. The column was kept at a constant 20°C because temperature influences anion-exchange separation. As with the previous method, an alkaline mobile phase with NaOH was used. Recoveries for this method were approximately 98%. The relative standard deviation of injections over the course of the experiment were determined to be 4.2% or lower indicating that the electrode was not fouling to an appreciable extent.
High Performance Liquid Chromatography

The term high performance liquid chromatography (HPLC) came about to describe the performance of separation achieved by high pressure liquid chromatography. As the name implies, the eluent is pushed through the stationary phase by a pump at high pressure, for typical analysis, at approximately 1500 psi. HPLC, based on partition process, is divided into two classes, normal phase and reversed phase HPLC (RP-HPLC). Normal phase HPLC is the less common technique. It operates under the principle of using a highly polar stationary phase coupled with a nonpolar mobile phase. Reversed-phase HPLC is simply the opposite, it uses a non-polar stationary phase with a polar mobile phase. The stationary phase for RP-HPLC is most commonly a long-chain hydrocarbon chemically bonded onto a solid support such as silica. The mobile phase for the reversed-phase technique is often methanol, water, acetonitrile, or a mixture thereof.

Reversed-Phase HPLC

Reversed-phase HPLC is the most common HPLC technique used. Approximately 75% of all HPLC analyses carried out are performed by RP-HPLC (15). This is due to its applicability to so many compounds of analytical interest. For analyzing compounds of an organic nature, RP-HPLC is the most logical choice because the stationary phase is non-polar and will have the greatest interaction with a non-polar organic analyte.

Reversed-phase chromatographic systems use a non-polar stationary phase. This stationary phase is most commonly an octyl C8, eight carbon chain, or octadecyl C18,
eighteen carbon chain. The longer these carbon chains are, the more non-polar the stationary phase. The analyte will have affinity for the stationary phase depending upon its polar character compared to the polarity of the stationary phase. For the typical reversed-phase system the more polar the analyte, the more quickly it will elute from the column. As the analyte of choice becomes increasingly non-polar, its affinity for the stationary phase will increase, and, therefore, the retention will increase.

For reversed-phase chromatography, the mobile phase is polar in nature. The most common mobile phases for reversed-phase consist of water, methanol, and acetonitrile. The mobile phase compositions can be varied to optimize the interaction between the analyte and the stationary phase. Methanol and water are very common choices because they are relatively inexpensive, highly pure, and HPLC grade varieties can be easily purchased. The mobile phase can be further modified by altering its pH and adding surfactants for improved resolution depending upon the analyte and sample matrix of interest.

**Detectors**

Detectors used with HPLC include those based upon electrochemical, fluorescence, UV-Visible (UV-Vis), and refractive index phenomena. However, among these, UV-Vis and refractive index detectors are the most commonly used. The detector of choice should offer good sensitivity. Modern detectors have sensitivities in the range of $10^{-8}$ to $10^{-15}$ g solute/s (15). The detector should also have good stability and reproducibility, a linear response that extends over several orders of magnitude, short response time, highly rugged and ease of use, and be non-destructive to the sample being analyzed (15).
Refractive Index Detectors. The refractive index detector is one of the most widely used and readily available detectors for HPLC. The refractive index detector is a bulk property detector which relies upon the presence of a solute to cause a change in refractive index of the measured solvent with reference to a pure solvent. This detector suffers from the disadvantages of low sensitivity, no selectivity, and that its response is affected by many factors which can alter the refractive index of the solvent.

Refractive index detectors, despite their disadvantages, are commonly used for carbohydrate analysis. However, it is important to note that care must be taken to ensure that the solvents used in the mobile phase are clean and pure and that the temperature and pressure of the system be carefully controlled. Without taking these precautions, the response is likely to be inaccurate with some change in the refractive index coming from temperature and pressure fluctuations and/or solvent impurities.

UV-Visible Detectors. The UV-Vis absorption detector is another common detector used in HPLC. Compared to other detectors, it is inexpensive and is readily available in most analytical laboratories. UV-Vis offers the advantages of good sensitivity, low cost applicability to a wide range of analytes and is relatively insensitive to changing conditions. Compared to a refractive index detector, the sensitivity of UV-Vis is greater, and it offers very good selectivity. Mobile phases for these detectors should however be of high purity and have very low or no absorbance in the chosen detection wavelength in order to avoid background absorption and interference.

UV-Vis detectors, while applicable to a wide range of analytes, are not usually the first choice for the detection of sugars. Mono- and disaccharides typically only absorb strongly in the 190-210 nm wavelength region. With modern instruments, sugars can be
detected in this region, but selectivity is a great concern. In this region, most organic compounds also absorb strongly. Therefore, only samples with very simple sample matrices can be accurately analyzed in this wavelength region. Derivatives may, however, be prepared in order to shift or allow absorbance at longer wavelengths where selectivity can be improved.

One such derivative that may be used to modify absorption by carbohydrates is that of para-aminobenzoic acid. Reductive amination with p-aminobenzoic acid leads to ring opening and the addition of p-aminobenzoic acid to the anomeric carbon of reducing sugars (26). Because this reaction only works with reducing sugars, selectivity is also improved. Reducing sugars can be determined in the presence of sucrose without interference. The added group also allows for detection wavelength range from 300nm to 360 nm, thus leading to further improved selectivity.

Chromatographic Columns

A wide variety of chromatographic columns are available commercially for varied applications and prices. Typically, guard columns are used to protect and extend the life of the primary analytical column. These small and short guard columns prevent contaminants and small solid particles from entering the analytical column for separation.

The primary columns used for separation of the analyte may vary in length, polarity, and particle size. The most common columns for RP-HPLC are the ones with C-8 (octyl) or C-18 (octadecyl) functional groups. Both are non-polar, long-chained hydrocarbons of eight and eighteen carbons in length, respectively. The C-18 is the more popular of the two. The C-18 is of course, the more non-polar of the two and, therefore, will increase the retention time of non-polar, organic analytes. Both columns are quite
inexpensive, approximately $300, and can be used with economical mobile phases, typically mixtures of methanol and water, thus adding to their appeal.

Ion-exchange is another type of column readily available. Their operation is based upon the attraction of opposite charges where ions of unlike charge bind with one another due to electrostatic attraction. These ionic interactions are used to separate components of the analyte sample based upon differences in their average charge. Ion-exchange resins with fixed negative charges are termed cation-exchange because the positively charged ions, cations, can only leave the resin when exchanged with other cations from the mobile phase. Alternatively, resins with fixed positive charges are termed anion-exchange resin.

Specialty columns developed for more specific applications are also available. These columns typically have, attached to the substrate, functional groups that have a strong affinity to specific analytes. As is to be expected, these columns are typically more expensive due to their specific and complex nature. Carbohydrate columns, for example, may be sold for approximately $1000 and upwards. These specialty columns may also require more specialized and unique mobile phases to prolong the column life and achieve adequate resolution. Unfortunately, these mobile phases may also be more expensive and/or less environmentally friendly. Despite the expense, specialty columns can be quite selective and offer very good resolution for an otherwise difficult separation.

**Efficiency and Resolution**

Theoretical plate count and plate height are terms used to describe the efficiency of a chromatographic column. These terms arose from a study by Martin and Synge where they described the chromatographic column as similar to a distillation column that
consisted of many narrow layers or theoretical plates (15). At each plate, it was postulated that equilibrium of the analyte between the stationary and mobile phases was established. Actually, the equilibrium state is never reached since the mobile phase is flowing at a relatively high speed. However, the term, theoretical plates, is still used for column efficiency.

Plate height, $H$, and plate count, $N$, are related by the expression,

$$N = \frac{L}{H} \quad (1)$$

where $L$ is the column length. Efficiency increases as the plate count increases and the plate height decreases. Variables that affect column efficiency, and thus plate height, include the linear velocity of the mobile phase, diffusion coefficient of the analytes in the mobile phase, diffusion coefficient in the stationary phase, retention factor, diameter of packing particle, and thickness of the stationary phase on solid support (15).

The van Deemter equation is a mathematical approximation of the efficiency of chromatography columns. The expression is:

$$H = A + \frac{B}{\mu} + C\mu = A + \frac{B}{\mu} + (Cs + Cm)\mu \quad (2)$$

Where, $H$ is the plate height in centimeters, $\mu$ is the linear velocity of the mobile phase in cm/sec, and A, B, and C are related to multiple flow paths, longitudinal diffusion, and mass transfer between phases, respectively (15). The C term can be separated into two parts, one related to the stationary phase, $Cs$, and one related to the mobile phase, $Cm$.

The A term, the multipath term, from equation 2, is related to zone broadening arising from the various paths by which a molecule or ion can move through a packed column (15). The length of the various pathways may differ significantly, and thus varies the time that the species stays on the column. This is sometimes referred to as eddy
diffusion, and it is directly proportional to the particle size of the stationary phase packing material (15).

The longitudinal diffusion term, $B/\mu$, is a band broadening effect in which solutes diffuse from the center of a flow zone to the more dilute regions outside the center of the zone (15). The longitudinal diffusion is inversely proportional to the mobile phase velocity because when the mobile phase velocity is higher the analyte is on the column for a shorter period of time. Therefore, diffusion from the center to the edges of the band has less time to occur.

Two mass transfer coefficients, $C_s$ and $C_m$, are used because the equilibration of the analyte between the mobile and stationary phases takes time to occur, and the column is never within complete equilibrium conditions. Band broadening due to mass transfer occurs because the streams of the mobile phase and the stationary phase have finite widths. Thus, time is required for the solute molecules to diffuse from the inside of the mobile and stationary phases to the exterior interface where interaction can occur (15).

Column resolution is the measure of its ability to separate two analytes. Resolution between two bands is defined as

$$\text{Resolution} = R_s = (V_{R2} - V_{R1}) / \left[ \frac{1}{2} (W_2 + W_1) \right] = \text{peak separation/peak width} \quad (3)$$

The denominator represents the average of the two baseline widths, and the numerator is the peak separation. A resolution value of 1.5 gives nearly complete separation of peaks with only about 0.1% overlap. At a value of 1 the points of two triangles that approximate the peaks just touch at the baseline, and at a resolution of 0.5 the peaks are significantly overlapping (19).
The principal difference between column efficiency and resolution is that efficiency describes band spreading. Ideally the observed peak should be narrow and thin. The efficiency is primarily influenced and determined by column construction, dimensions of the packing, and the velocity of the mobile phase (19). Resolution is a measure of the degree of separation. It is determined by choice/type of stationary phase, mobile phase, temperature, and the length of the column (19). The greater the interaction between the mobile phase, analyte, and the column the better for both column efficiency and resolution.

Proposed Research

Carbohydrates play a vital role in the human diet. They are an economical and readily available source of food energy. Lactose is among the most common carbohydrates found in foods. It is often thought of simply as “milk sugar”. It is definitely present in dairy products but also in a wide variety of other foods including baked goods, beverages and beverage mixes, and prepared or processed meats. Lactose can however present difficulties to those who are lactose intolerant due to a low level of lactase. Lactase is the enzyme that breaks down lactose into its two monosaccharide units, glucose and galactose, during digestion. Lactose intolerance is especially common in Asian and African cultures. This disorder is most common among adults, but it also occurs in infants and children. Lactose intolerance is even a greater concern for infants because for the first part of life, nutritional energy comes solely from milk. Lactose is the primary source of carbohydrate energy found in milk and infant formula. The quantitation of lactose is extremely important to protect the health of lactose intolerant individuals especially babies and young children.
The analysis of carbohydrates in foods presents a challenge. The common sugars found in foods are very similar in structure and chemistry, therefore, making selectivity difficult. Often, these sugars occur together in many foods. It is common for a food to contain both lactose and sucrose or lactose and fructose for example. The challenge then becomes to exploit the very slight differences in structure to selectively determine each sugar. Also, the sample matrix of food samples, especially infant formula, can be quite complex. Unfortunately, the samples are not merely a combination of sugars in solution. In the case of infant formula, fats and proteins are present in large amounts to supply the energy and developmental needs of infants.

Reversed-phase high performance liquid chromatography is a widely used tool in the analysis of foods. Currently, the most common detection method for simple sugars is refractive index. As discussed earlier, in Chapter 2, refractive index detection offers no selectivity and low sensitivity. Also the sample and solvents used must be very pure, and many conditions, such as temperature and pressure, must be carefully controlled to avoid inaccurate changes of refractive index due to factors other than the sample itself.

UV-Visible detection is desirable as it offers the selectivity and sensitivity lacking in refractive index detection. However, this technique requires making a derivative of the sugar in order to achieve absorption at higher wavelengths to avoid interference from other organic compounds thus increasing the selectivity of the technique.

The focus of this research project is to develop a sensitive, selective method of analysis for lactose using reversed-phase high performance liquid chromatography. The proposed objectives of this project are outlined and described below.
1. Detect lactose and other simple sugars using a UV-Vis diode array detector. Derivatives of lactose through reductive amination with p-aminobenzoic acid will be made in order to detect it at a suitable wavelength range, 300-360 nm, to achieve selectivity.

2. The procedure for forming the above mentioned derivative will be optimized to achieve maximum signal with minimal side reactions while keeping reaction time at a reasonable period.

3. An economical and benign mobile phase will be established for use with a reversed-phase column. The effects of surfactants and pH on resolution will also be evaluated.

4. A reversed-phase column will be chosen and compare its performance to that of a specialty carbohydrate column.

5. The figures of merit will be used in order to determine the validity of the analytical method developed.

6. The HPLC method will then be applied to commercial lactose containing samples, especially those intended for infants.

7. Finally, the HPLC method will be compared to an established method, the alkaline ferricyanide method.
Experimental Procedures

The following sections describe the experimental procedures performed in order to develop and establish the validity of a RP-HPLC method for the quantitative determination of lactose. This method is evaluated based upon selectivity, reproducibility, accuracy, and its applicability to the determination of lactose in commercial food samples, especially those intended for infant and toddler nutrition. To validate the method, linearity, reproducibility, and recovery studies were conducted. To further confirm our results, the alkaline ferricyanide method was also applied, and the results obtained from the two methods were compared.

Reagents

The list of reagents used throughout the project, their grades, and suppliers are given below.

Deionized water was obtained from Continental Water System (Millipore, Bedford, MA).

HPLC grade methanol and acetonitrile, 99.9 % purity, and DMSO, 99 % purity, were obtained from Fisher Scientific (Fairlawn, NJ).

Sodium carbonate, 100 % purity, ferric ammonium sulfate, 99.5 % purity, potassium ferricyanide, 99.96 % purity, and potassium cyanide, 99.7 % purity, were all obtained from Mallinckrodt Chemical (Paris, KY).
Sodium cyanoborohydride, 95 % purity, p-aminobenzoic acid, 99 % purity, cetyltrimethylammonium bromide (CTAB), 95 % purity, were all obtained from Aldrich Chemical Company (St. Louis, MO).

Lactose, glucose, fructose, and sucrose, 99 % purity, and 1-octanesulfonic acid, 95 % purity, were obtained from Sigma (St. Louis, MO).

Glacial acetic acid, 99.8 % purity, was obtained from Merck (Rahway, NJ).

Triton X-100, scintillation grade, was obtained from Eastman Chemical Company (Kingsport, TN).

**Instrumentation**

A Perkin Elmer HPLC system, generously donated by Eastman Chemical Company, was used for analysis. The system included a model ISS 200 auto-sampler, a series 410 LC pump, a 235C diode array detector, and a Dell personal computer with Turbochrom Navigator software. All the components were connected using a PE Nelson 600 Link Box. The primary column used for analysis was a Zorbax 4.6 mm x 25 cm C8 column. Zorbax 4.6 mm x 25 cm C18 and a Waters high performance carbohydrate cartridge 4.6 mm x 250 mm were also tried. A Waters Nova-Pak C18 guard column was also present.

A UV-Visible spectrophotometer was used for the ferricyanide method for comparison to results obtained from the HPLC method. The UV-Vis Spectronic 301 was manufactured by Milton Roy.
Preparation of Reagents and Stock Solutions

The following reagents were prepared as described below. The reagents for the derivatization reaction, reductive amination, were prepared as described by Fisher, Meyer, and Raba (21). The reagents associated with the alkaline ferricyanide method were prepared as described in Determination of Food Carbohydrates (27).

1. P-aminobenzoic acid solution (0.35M): 480 mg of p-aminobenzoic acid dissolved in 70:30 mixture of DMSO: Glacial acetic acid in a 10-mL volumetric flask.

2. Alkaline cyanide solution: 0.53 % (w/v) sodium carbonate containing 0.065% (w/v) potassium cyanide. The 0.53% sodium carbonate was prepared by diluting 265 mg of sodium carbonate to 50 mL with deionized water. The
0.065% potassium cyanide solution was prepared by diluting 32.5 mg of potassium cyanide to 50 mL with 0.53% sodium carbonate solution.

3. Potassium ferricyanide solution 0.05% (w/v): 25 mg of potassium ferricyanide was dissolved and diluted to 50 ml. with deionized water.

4. Ferric ammonium sulfate solution: 150 mg of ferric ammonium sulfate diluted to 100 mL with 0.025 M sulphuric acid.

Preparation of Standard Solutions

Stock standard solutions were prepared for glucose, fructose, sucrose, and lactose. Each sugar solution was prepared to be 0.010 M. Glucose, fructose, lactose, and sucrose, 18 mg, 18 mg, 34.2 mg, and 36.0 mg, respectively, were added to separate 10-mL. volumetric flasks and diluted with deionized water. Each solution was stoppered and sonicated until completely dissolved. These solutions were then stored in glass vials in the refrigerator. The solutions were prepared fresh monthly.

Preparation of Commercial Samples

Commercial samples of infant formula liquid concentrate and in powder-form, yogurt-juice blends for infants and toddlers, and non-fat dry milk were obtained from local stores. The lactose content was calculated based upon the manufacturer’s nutritional information per serving. Each sample was prepared to a concentration approximately equal to the concentration of the lactose standard, 0.010 M or 3.60 mg/mL.

For all samples, except the non-fat powdered milk, the fat was extracted in order to prevent interference during derivatization. The sample was dissolved and placed into 125 mL separatory funnel. Approximately 20 mL of ethyl ether was added. The solution
was shaken while opening the valve periodically to allow the gas to escape. The aqueous
and organic layers were allowed to separate. The bottom layer, the aqueous layer, was
collected. The extraction was repeated until no more fat was visible with the separation.

**Derivatization of Sugars**

The procedure for the preparation of p-aminobenzoic acid derivatives of sugars by
reductive amination was performed according to the method described by Fischer,
Meyer, and Raba (21). The reaction mixtures consisting of 200 µL of sugar solution,
50 µL of p-aminobenzoic acid solution (PABA), and 10 mg of sodium cyanoborohydride
were added to separate glass vials and loosely capped. The mixture was heated in a 60°C
water bath for 45 minutes. Upon cooling, the contents of each reaction vial were
transferred to 10-mL volumetric flasks. The solutions were then diluted to volume with a
mixture of methanol: water (70:30). The derivatives were then stored at room
temperature until injection for HPLC analysis.

**HPLC Conditions**

All HPLC separations were performed under isocratic conditions with a flow-rate
of 0.5 mL/min. The mobile phase was composed of 85:15 methanol: water mixture with
the pH adjusted to 5.5 with glacial acetic acid. Each component of the mobile phase was
thoroughly degassed for approximately 30 minutes with helium gas. The two solvents
were mixed by the Perkin-Elmer series 410 LC pump. The diode array detector
wavelength range was set to 300-360 nm. The injection volume was 10 µL.

**Data Analysis**

Data were collected directly from the HPLC system with Turbochrom Navigator
software. Statistical analysis of the data generated in Turbochrom was performed using
Microsoft Excel software for Microsoft Windows XP. After plotting the data, regression analysis was performed to produce the equation of the regression line and also the correlation coefficient. Mean, standard deviation, and relative standard deviation were also calculated using a Microsoft Excel spreadsheet for replicate analysis of the same sample and analysis conditions.

The data collected during the described experiments are discussed in the sections to follow. In order to establish reproducibility of results, samples were prepared, analyzed, and injected in triplicates. The mean, standard deviation, and relative standard deviation were then calculated for each data set.

Results and Discussion

In the following sections, the optimization of the proposed procedures and experimental conditions will be explored and discussed. The proposed method of analysis of lactose will be evaluated based upon selectivity, sensitivity, reproducibility and accuracy. Each of these figures of merit was determined by reproducibility studies, linear dynamic range, and recovery studies. The applicability to commercial products is also evaluated. Finally, the proposed analytical method is compared to an accepted analytical method, the alkaline ferricyanide method, by comparison of the lactose concentration determined by both methods in commercial samples.

Optimization of the P-aminobenzoic Acid Derivatization of Sugars

The procedure used for the preparation of derivatives of mono- and disaccharides by reductive amination with PABA was adapted from the method proposed by Fisher, Meyer, and Raba (21). As discussed previously, it is necessary to prepare a derivative of the sugars in order to achieve appreciable UV absorption at a suitable wavelength that
minimizes interference from other organic compounds. The derivatives prepared by this method absorb strongly in the 300-360 nm wavelength region with minimal interference. The goal of the optimization of the derivatization procedure is to react the mixture as completely as possible with minimal of side-reactions.

The Fisher, Meyer, and Raba method proposes using a large excess of the PABA reagent, 500 µL of 0.35 M concentration (21). It was determined, however, that the excess of unused reagent produces a large peak in the chromatogram that nearly overshadows the peak produced by the analyte by comparison. It was found, by experimenting with smaller volumes of PABA, a lesser amount of it could be used, thereby decreasing the reagent peak size while still obtaining a reaction product of the same magnitude.

The method proposed in the literature also suggests a reaction time of 10 minutes at 60°C. Experiments were conducted to determine if this reaction time was able to achieve the highest level of completion for the reaction of lactose. The reaction was carried out for 15, 30, 45, and 60 min. The reproducibility of the reaction product as well as the appearance of extraneous peaks due to side-products at these time intervals was compared. The results of this set of experiments are shown in Table 1.

In one trial of the 45-minute reaction time, it appeared that the reaction did not go to completion. Additional trials with the 45-minute reaction time were made. The results of this set of experiments are given in Table 2.
Table 1. Results of Lactose Determination at Different Reductive Amination Reaction Times.

<table>
<thead>
<tr>
<th>Reaction Time (min.)</th>
<th>Lactose Peak Area</th>
<th>Mean Area</th>
<th>Standard Deviation</th>
<th>% R.S.D</th>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>2208778</td>
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<td>7.87</td>
</tr>
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<tr>
<td></td>
<td>3537567</td>
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</tr>
</tbody>
</table>

Note: The reactions were carried out in a water bath held at 60°C. The reaction mixture consisted of 200 µL of 0.010 M lactose solution, 50 µL PABA reagent, and 10 mg of NaCNBH₃. The retention time for lactose was 4.9 minutes.
Table 2. Results of Lactose Determination at 45 Minute Reductive Amination Reaction Time.

<table>
<thead>
<tr>
<th>Lactose Peak Area</th>
<th>Mean Area</th>
<th>Standard Deviation</th>
<th>% R.S.D</th>
</tr>
</thead>
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</tbody>
</table>

Note: The reactions were carried out in a water bath held at 60°C. The reaction mixture consisted of 200 µL of 0.010 M lactose solution, 50 µL PABA reagent, and 10 mg of NaCNBH₃. The retention time for lactose was 4.9 minutes.

From the comparison of the peak areas for the lactose derivative peak at 4.9 min. retention time, it was determined that the reaction time of 60 minutes produced an additional shoulder peak adjacent to the peak corresponding to excess PABA. It was, therefore, determined that 60 minutes was not the best reaction time as it affects the resolution of the sugar peaks.

As shown in Figure 2, the resolution between the lactose peak, at 4.9 minutes, and the excess PABA, at 5.3 minutes, is decreased. The two peaks are not baseline resolved as they are at the 45 minute reaction time. The peak shape of the excess PABA is not as good. The peak is split into a shouldered peak, with the shoulder at 5.47 minutes.
Figure 2. HPLC chromatogram of lactose standard after 60-minute reaction time at 60°C. The reaction mixture consisted of 200 \( \mu \text{L} \) of 0.010 M lactose, 50 \( \mu \text{L} \) of 0.35M PABA, and 10 mg of sodium cyanoborohydride. Separation was achieved with 0.50 mL/min of a 85:15 (methanol:water) mobile phase. The detection wavelength range was 300-360 nm.

The reaction times of 15, 30, and 45 minutes all showed clean chromatograms with peaks due only to the lactose derivative and the excess PABA reagent. The 45-minute reaction time was chosen because it allowed for a more complete reaction.
compared to those carried out for 15 and 30 minutes. The result obtained from the 45-minute reaction time was still quite reproducible with a relative standard deviation of 12.9% compared to 6.80% and 7.87% for 30 minutes and 15 minutes, respectively. The chromatograms for the various reaction times are similar, and only the chromatogram corresponding to the 45-minute reaction time is shown in Figure 3.

![HPLC chromatogram of lactose standard after 45-minute reaction time at 60°C.](image)

**Figure 3.** HPLC chromatogram of lactose standard after 45-minute reaction time at 60°C. The reaction mixture consisted of 200 µL of 0.010 M lactose, 50 µL of 0.35M PABA, and 10 mg of sodium cyanoborohydride. Separation was achieved with 0.50 mL/min of a 85:15 (methanol:water) mobile phase. The detection wavelength range was 300-360 nm.

**Selectivity of Derivatization Reaction for Reducing Sugars**

It is beneficial to prepare a derivative of the sugars of interest selective only to particular sugars. This eliminates and/or reduces interference from other sugars that may be present within the food sample matrix. This is especially important because often
times there is more than a single carbohydrate source present. Sucrose and fructose may both be present in fruit flavored beverages for example.

The previously described method for the derivatization of sugars by reductive amination with PABA was applied to common mono- and disaccharides present in food samples. These included sucrose, fructose, glucose, and lactose. Results indicate that the reaction does not proceed to an appreciable degree for fructose and sucrose. As shown in Figures 5 and 6, the chromatograms for sucrose and fructose standard solutions at the 45-minute reaction time do not indicate a peak for the presence of the derivative other than the excess PABA peak. A peak in the HPLC chromatogram was, however, observed for both lactose and glucose when reacted under the conditions previously discussed. The lack of reactivity of sucrose can be explained by the fact that sucrose is a non-reducing sugar. It does not have a free anomeric carbon for the lone pair of the amino group to attack. Fructose is a ketose rather than an aldose sugar. The ketone group makes the anomeric carbon less reactive to reductive amination. The chromatograms for the derivatives of fructose, glucose, and sucrose are displayed in Figures 4-6. The lactose derivative corresponding to the same reaction conditions was shown in Figure 4. The chromatograms showed that the retention time for lactose was 4.9 minutes, and the glucose retention time was 5.0 minutes. These two sugars were adequately separated.
Figure 4. HPLC chromatogram for the PABA derivative of fructose after 45 minutes at 60°C. The reaction mixture consisted of 200 µL of 0.010 M fructose, 50 µL of 0.35M PABA, and 10 mg of sodium cyanoborohydride. Separation was achieved with 0.50 mL/min of a 85:15 (methanol:water) mobile phase. The detection wavelength range was 300-360 nm.
Figure 5. HPLC chromatogram for the PABA derivative of glucose after 45 minutes at 60°C. The reaction mixture consisted of 200 µL of 0.010 M glucose, 50 µL of 0.35M PABA, and 10 mg of sodium cyanoborohydride. Separation was achieved with 0.50 mL/min of a 85:15 (methanol:water) mobile phase. The detection wavelength range was 300-360 nm.
Figure 6. HPLC chromatogram for the PABA derivative of sucrose after 45 minutes at 60°C. The reaction mixture consisted of 200 µL of 0.010 M sucrose, 50 µL of 0.35M PABA, and 10 mg of sodium cyanoborohydride. Separation was achieved with 0.50 mL/min of a 85:15 (methanol:water) mobile phase. The detection wavelength range was 300-360 nm.

Selection of Stationary Phase

The choice of the correct chromatographic column with the right stationary phase is vital to achieve good resolution. For the separation of PABA derivatives of sugars, Fishcher, Meyer, and Raba used C18 and anion exchange columns (21). The column
chosen for their work was the C18 with a buffered combination of methanol and water for the mobile phase (21).

For this project, three different columns were evaluated, Zorbax 4.6 mm x 25 cm C8, Zorbax 4.6 mm x 25 cm C18, and Waters 4.mm x 25 mm High performance Carbohydrate Cartridge. The initial column used was the Waters Carbohydrate Cartridge which was silica modified with amino propylsilane. This column is frequently used to separate underivatized carbohydrates in conjunction with refractive index detection.

To evaluate the suitability of each column for the separation of glucose and lactose derivatives, the reaction was conducted under the previously described conditions with a vessel containing both glucose and lactose. The derivatives were injected. Results indicate that the affinity of the derivatives for the carbohydrate column was too strong. Peaks would be observed at random times indicating the possibility that the derivatives were being held on the column longer than the expected run time and were eluted during the subsequent injections. It was, therefore, determined that the Waters Carbohydrate Cartridge was not suitable for the separation of the derivatives of glucose and lactose.

The Zorbax C18 column was also tried with a mobile phase of 75:25 (methanol: water). With this column, all three species present, glucose, lactose, and excess PABA were eluted quickly in less than 3 minutes at a flow rate of 1.0 mL/min. As a result of rapid elution, resolution was very poor. The chromatogram appeared as one large shouldered peak with virtually no resolution between the excess PABA and the sugar derivatives. Figure 7 is the chromatogram that was obtained and it clearly illustrates that the resolution is poor.
The separation on the C8 column was much improved compared to the previous two columns tried. Resolution between glucose, lactose, and excess PABA was achieved. Elution time was still less than 3 minutes using the same 75:25 (methanol:water) mobile phase at a 1 mL/min flow rate. Figure 8 illustrates that the peaks corresponding to glucose at 2.58 minutes and lactose at 2.49 minutes are resolved and the excess PABA is eluted later at 2.90 minutes. This is due to increased polarity of the C8 column compared to the C18 column. The interaction of the derivatives with the more polar stationary is thus increased leading to improved resolution.
Figure 8. HPLC chromatogram of glucose and lactose derivatives and excess PABA derivative using C8 column with 75:25 (methanol:water) mobile phase at a flow rate of 1.0 mL/min.

**Optimization of Mobile Phase**

To further improve the resolution of glucose and lactose derivatives, additional compositions of the mobile phase containing methanol and water were varied. While still maintaining the flow rate of 1 mL/min, the ratios of methanol to water were adjusted as
follows, 85:15, 90:10, and 100% methanol. The best resolution was obtained with the 85:15 mobile phase. There was little change by using the 90:10 methanol: water mobile phase, with resolution being approximately the same as the 85:15 mobile phase. With the use of 100% methanol, resolution suffered. A small amount of water is needed in order to aid in the elution of the sample components. Without the water, the components are not eluted as quickly.

**Optimization of Flow Rate.** The resolution was further improved by lowering the flow rate to .5 mL/min. By lowering the flow rate and maintaining the solvent composition of 85:15 methanol: water, the interaction between the stationary phase and the analytes is increased thus leading to improved resolution. Figure 8 represents the flow rate of 1.0 mL/min. Figures 9-11 represent the chromatographic runs showing the improvement in peak resolution. Note also that elution time is increased to 4.9 and 5.0 minutes for the lactose and glucose, respectively, and 5.5 min. for the excess PABA.

![Figure 9. HPLC chromatogram of lactose and glucose PABA derivatives at a mobile phase flow rate of 0.5 mL/min consisting of 75:25 (methanol:water)](image-url)
Figure 10. HPLC chromatogram of lactose and glucose PABA derivatives at a mobile phase consisting of 100% methanol at a flow rate of 0.5 mL/min consisting of 75:25 (methanol:water).

Figure 11. HPLC chromatogram of lactose and glucose PABA derivatives with a mobile phase consisting of 85:15 (methanol:water) at a flow rate of 0.5 mL/min. Lactose has a retention time of 4. min. Glucose has a retention time of 5.0 minutes.
Effects of Adding Surfactants to the Mobile Phase

Surfactants, long non-polar hydrocarbon chains with polar groups at the head of the chain, are often introduced into the mobile phase (12). It is believed that by adding surfactants one can improve resolution by increasing the interaction of the mobile and stationary phase by increasing the surface area of their interaction. Cheetham and Tang reported shorter retention times of oligosaccharides when treating their C18 column with cetyl trimethylammonium bromide or Triton-X (12). The decrease in retention times by added detergents was a result of a reduction of the surface tension between the stationary phase and the mobile phase, which affected the transfer of the solute from the mobile phase to the stationary phase and vice versa (12).

In order to determine the effects of surfactants on the resolution of the glucose and lactose derivative peaks, three different surfactants were tried Triton X-100, 1-octanesulfonic acid (OSA), and cetyltrimethylammonium bromide (CTAB). These three were chosen based upon their differences. Triton X-100 is a non-ionic detergent with a mixture of p-tert-octylphenoxy-polyethoxyethanol oligomers (12). CTAB is a cationic surfactant compared to OSA which is an anionic surfactant.

Solutions of the three surfactants replaced water in the mobile phase. Solutions containing 1% (w/w) of surfactant in water were prepared. Mobile phase compositions of 85:15 methanol: surfactant solution were tried. Comparisons of these results are represented by Figures 12-14. From appearance of the chromatograms there is no significant advantage to adding surfactants. All four mobile phases show similar resolution between the glucose and lactose derivative peaks.
Figure 12. HPLC chromatogram of lactose and glucose PABA derivatives with a mobile phase consisting of 85:15 (methanol:CTAB)

Figure 13. HPLC chromatogram of lactose and glucose PABA derivatives with a mobile phase consisting of 85:15 (methanol:OSA)
Effects of pH of the Mobile Phase  The effects of pH on resolution were also explored. Kuster and Verhaar suggested a neutral mobile phase based upon their study of the retention behavior of carbohydrate oligomers. They used a pure water mobile phase with the pH adjusted to 2, 6.5, and 10. They suggested that resolution and retention time improved at a pH of 10, but they also noted that the improvement was not worth the risk of degradation of the stationary phase (17).

For the 85:15 methanol: water mobile phase, pH values were adjusted to 4, 5, and 7.5. A phosphate buffer was used to adjust and maintain the pH of 7.5. All others were adjusted by the addition of glacial acetic acid to the methanol, the largest component of the mobile phase. It should, however, be noted that the more appropriate way to refer to this pH would be apparent pH since it is not measured in a purely aqueous environment.
These pHs did not appear to have a significant effect upon the resolution or retention
times of the analytes. The pH value, therefore, was determined not as important as just
simply controlling the pH. The pH if not controlled could fluctuate depending upon the
methanol and water used, resulting in lack of reproducibility of the retention times. The
pH of the mobile phase was, therefore, adjusted and maintained at 5. The results of the
change of pH on the chromatographic determination of the sugars can be observed in
Figures 15-17.

At pH 7.5, represented in Figure 16, the retention times of glucose and lactose are
4.768 and 4.699, respectively. At the more acidic pH of 5, Figure 15, the retention times
of glucose and lactose are 5.0 and 4.9, respectively. Finally, the pH of 4 was
investigated. At the pH of 4, Figure 14, there is no difference in the retention times
compared to the pH of 5. Based upon these results, the effects of pH are restricted to
only the slight variation in retention time. There is effect on the resolution of the glucose
and lactose peaks. At the more acidic pH values, the retention time is slightly longer
compared to the pH of 7.5. Without pH control, the retention time varied between 4.7
and 5.0. This variation could lead to difficulties in peak identification in a mixture, and
therefore it was decided that pH control was important.
Figure 15. HPLC chromatogram of lactose and glucose derivatives with the mobile phase pH adjusted to 4.0 with glacial acetic acid. The mobile phase consists of 85:15 (methanol:water) at a flow rate of 0.5 mL/min.

Figure 16. HPLC chromatogram of lactose and glucose derivatives with the mobile phase pH adjusted to 5.0 with glacial acetic acid. The mobile phase consists of 85:15 (methanol:water) at a flow rate of 0.5 mL/min.
Reproducibility Studies

The reproducibility of any analytical method must be considered and evaluated before its application. These studies were first performed to determine the reproducibility of the proposed HPLC method for the determination of lactose. The reaction was carried out as previously described in a reaction vessel containing 200 µL of lactose solution, 50 µL PABA solution, and 10 mg of sodium cyanoborohydride six times. Each reaction mixture was injected in duplicates with the exception of the first trial which was injected in triplicates. The method was reproducible with regard to lactose as the relative standard deviation of the peak area was 4.96%. This is
considerably better than the result for lactose reported by Fisher, Meyer, and Raba (21). They reported a relative standard deviation of 13% for lactose (21).

Table 3. Reproducibility Data for the Lactose Standard Solution

<table>
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<tr>
<th>Trial</th>
<th>Peak area (first injection)</th>
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</tbody>
</table>

Note: The mean area was 1779559 with a standard deviation of 88186 and relative standard deviation of 4.96%.

The reproducibility of glucose and a glucose and lactose mixture were also evaluated. For these experiments the reactions were carried out four times and injected in duplicates. The relative standard deviation for glucose alone was 11.09%. Within the mixture, the relative standard deviations were 13.3% and 6.5% for glucose and lactose, respectively. It appears that the derivatization process for both glucose and lactose are slightly less reproducible within a mixture sample. This could be due to the lower amount of excess PABA reagent as the amounts of the sugars is increased, while the amount of PABA and other reagents remains unchanged.
Table 4. Reproducibility Data for the Glucose Standard Solution

<table>
<thead>
<tr>
<th>Trial</th>
<th>Area (injection 1)</th>
<th>Area (injection 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>744049</td>
<td>747058</td>
</tr>
<tr>
<td>2</td>
<td>720738</td>
<td>716078</td>
</tr>
<tr>
<td>3</td>
<td>684745</td>
<td>680905</td>
</tr>
<tr>
<td>4</td>
<td>550129</td>
<td>574546</td>
</tr>
</tbody>
</table>

Note: The mean area for the glucose peak area was 677281 with a relative standard deviation of 11.1%

Table 5a. Reproducibility Data for Glucose within the Mixture

<table>
<thead>
<tr>
<th>Trial</th>
<th>Area (injection 1)</th>
<th>Area (injection 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>307872</td>
<td>322732</td>
</tr>
<tr>
<td>2</td>
<td>320940</td>
<td>325360</td>
</tr>
<tr>
<td>3</td>
<td>362168</td>
<td>365690</td>
</tr>
<tr>
<td>4</td>
<td>252024</td>
<td>258366</td>
</tr>
</tbody>
</table>

Note: The mean area count for the glucose peak area within the mixture containing glucose and lactose was 314394 with a relative standard deviation of 13.3%
Table 5b. Reproducibility Data for Lactose within the Mixture

<table>
<thead>
<tr>
<th>Trial</th>
<th>Area (injection 1)</th>
<th>Area (injection 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85866</td>
<td>87285</td>
</tr>
<tr>
<td>2</td>
<td>81515</td>
<td>86822</td>
</tr>
<tr>
<td>3</td>
<td>84368</td>
<td>88097</td>
</tr>
<tr>
<td>4</td>
<td>74611</td>
<td>75224</td>
</tr>
</tbody>
</table>

Note: The average peak area for lactose within the mixture containing glucose and lactose was 82973 with a relative standard deviation of 6.5%.

**Linear Dynamic Range**

In order to examine the linear relationship of concentration and peak area of lactose, concentrations of lactose ranging from 0.018-0.36 mg/mL were prepared and analyzed in succession. Lactose standards for each concentration were derivatized in triplicates and injected in duplicates. The results obtained are summarized in Table 3.

The calibration curve is plotted and shown in Figure 18. The equation of the regression line is $y = 3 \times 10^7 x - 2 \times 10^6$ with an $R^2 = 0.9298$. The $R^2$ essentially means that 92% of the independent variable is explained by the dependent variable $x$. The correlation coefficient, $R = .96$, indicates a strong linear relationship between the peak area and lactose concentration. The lower limit of detection was estimated to be 0.06 mg/mL. At this point, the relationship was no longer as reproducible and no longer fit the linear relationship.
Table 6. Areas of Analyte Peak at 4.9 min.

<table>
<thead>
<tr>
<th>Lactose concentration (mg/ml)</th>
<th>Peak Area</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>.1802</td>
<td>1559920 1522508 1288681 1268913 1336381 1330137</td>
<td>1384423</td>
<td>124604</td>
<td>9.00</td>
</tr>
<tr>
<td>.2402</td>
<td>2187313 2168940 2447174 2302951 2512724 2428674</td>
<td>2341296</td>
<td>143611</td>
<td>6.13</td>
</tr>
<tr>
<td>.3275</td>
<td>10179605 9851610 9916413 9751230 9676699 9555562</td>
<td>9821853</td>
<td>216694</td>
<td>2.21</td>
</tr>
<tr>
<td>.3431</td>
<td>12929336 12359395 13020641 12526707 13154158 12374045</td>
<td>12727380</td>
<td>349129</td>
<td>2.74</td>
</tr>
</tbody>
</table>

Note: The reaction mixture consisted of 200 µL of lactose, 50 µL of 0.35 M PABA reagent, and 10 mg of sodium cyanoborohydride. The reaction proceeded for 45 minutes at 60°C in a water bath. Each reaction was performed in triplicates and injected in duplicates.
Application of the Proposed HPLC Method to Commercial Samples

In order to determine the applicability of the developed method to commercial food samples, eight samples were purchased from local stores for the determination of lactose. These samples included Parent’s Choice infant formula, Nestle Good Start infant formula, Enfamil powder infant formula with iron, Isomil Soy powder infant formula, Carnation non-fat dry milk, Gerber Banana Smoothie juice, Gerber Graduates Berry Smoothie juice, and Enfamil Lacto-free Lipil infant formula.

The food samples were prepared and the final concentrations in the solutions made according to the manufacturers reported amount of lactose per serving. The samples were prepared in more dilute forms to be appropriate for derivatization. For the infant formulas and juice samples with fat present, the fat was extracted with ethyl ether. This was done in order to reduce/eliminate interference by the fat present. The amount of
lactose present in the diluted sample was then compared to a calibration curve obtained by preparing lactose standards over the appropriate concentration range. The dilutions were then taken into consideration and the amount of lactose per serving was calculated. The results for Isomil Soy and Enfamil Lacto-free Lipil were below the limit of detection, as expected. These formulas are especially intended for lactose intolerant infants, and the manufacturers claim that they contain no lactose. These lactose-free formulas are primarily sweetened with sucrose.

The highest concentrations of lactose were found in the three infant formulas, Parent’s Choice, Nestle Good Start, and Enfamil. The higher lactose levels are present in order to meet the carbohydrate energy needs of the developing infant. The lowest concentrations were determined in the Gerber smoothie juice blends containing yogurt. This is expected because these drinks are primarily fruit juice rather than dairy. It can be noted however, that the smoothie juices contain fructose which did not interfere with the determination of lactose because fructose does not react by reductive amination with PABA.

In general, the infant formulas tested by the HPLC method had a lower lactose content than was reported by the manufacturer. This was especially true with the Enfamil powder formula. The lactose content determined by HPLC was 33.9 mg/mL compared to the reported 70.67 mg/mL. The difference can partially be explained by the manufacturer’s method of reporting nutritional content. Results are reported as an average content over several batches of product. Inevitably, there is variation in the product and nutritional content of the product. The additional difference between the powder formula’s lactose content versus the manufacturers results may be due to
moisture absorption by the powder, and with the powder mixing may not be as uniform because the powder content is less homogeneous than the liquid formula.

The lactose content of fruit juice drinks is found to be higher than the reported concentration of the manufacturers. The lactose concentrations determined by the HPLC method were 7.75 mg/mL and 7.0 mg/mL for the Gerber Banana smoothie and the Gerber Graduates Berry smoothie, respectively. This is in comparison to 4.14 mg/mL and 2.1 mg/mL reported by the manufacturer. This is most likely due to an interfering species or other sugars present within the sample matrix.

Table 7. Lactose Content of Commercial Samples Determined by the HPLC Method and the Manufacturers’ Label.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lactose (mg/mL) by HPLC</th>
<th>Lactose (mg/mL) by Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent’s Choice Infant</td>
<td>61.5</td>
<td>70.67</td>
</tr>
<tr>
<td>Formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nestle Good Start</td>
<td>57</td>
<td>74.67</td>
</tr>
<tr>
<td>Formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enfamil powdered</td>
<td>33.9</td>
<td>70.67</td>
</tr>
<tr>
<td>formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carnation non-fat dry</td>
<td>42.72</td>
<td>50</td>
</tr>
<tr>
<td>milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerber Banana</td>
<td>7.75</td>
<td>4.14</td>
</tr>
<tr>
<td>Smoothie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerber Graduates Berry</td>
<td>7.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Smoothie</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8. Results of the Determination of Lactose Present in Commercial Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area</th>
<th>% R.S.D.</th>
<th>Amount per suggested serving (mg/mL) by HPLC method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent’s Choice Infant Formula</td>
<td>3449820 5449303 4714142 4366910 5230091 5186467</td>
<td>15.7</td>
<td>61.5</td>
</tr>
<tr>
<td>Nestle Good Start infant formula</td>
<td>450863 484342 485467 482538</td>
<td>3.5</td>
<td>57</td>
</tr>
<tr>
<td>Enfamil powder infant formula</td>
<td>3271079 3290909 3569029 3405431 2908892</td>
<td>7.4</td>
<td>33.9</td>
</tr>
<tr>
<td>Carnation dry milk</td>
<td>6504971 6419035 6782357 6607458 6411957 6453630</td>
<td>2.2</td>
<td>42.72</td>
</tr>
<tr>
<td>Gerber Banana Smoothie</td>
<td>210858 202303 224539 246824 229617</td>
<td>7.7</td>
<td>7.75</td>
</tr>
<tr>
<td>Gerber Graduates Berry Smoothie</td>
<td>4501837 4482363 4340485 4179267</td>
<td>3.4</td>
<td>7</td>
</tr>
</tbody>
</table>
Recovery Studies

In order to evaluate the accuracy of the method, recovery studies were performed. A standard calibration curve was generated with concentrations over the 0.009 mg/mL - 0.36 mg/mL range. This curve was used to determine the lactose concentration of recovery samples. In the first study, the commercial Carnation non-fat dry milk was used. 2.875 g of powder, corresponding to 1.5 g of lactose was dissolved in 500 mL of deionized water. This corresponded to a concentration of approximately 3.0 mg/mL of lactose. To this sample, the 0.010 M lactose standard was added to the reaction mixture in the following volumes: 0, 25 µL, 50 µL, 30 µL, and 60 µL. This corresponded to the addition of 0, 0.12 mg/mL, 0.18 mg/mL, 0.14 mg/mL, and 0.20 mg/mL lactose respectively. Each reaction was performed and injected in triplicates. The difference in the absorbance should correspond to the concentration difference on the calibration curve. The results are given in Table 9.

An additional recovery study was performed with Nestle Good Start infant formula. Once again, the infant formula was prepared as previously described in an approximately 3 mg/mL concentration to be appropriate for analysis. To this solution spikes of 50 and 100 µL of 0.010 M lactose standard were added. The difference in absorbance was then compared to the calibration curve. The results for these studies are summarized in Table 10.

A recovery study was also performed on lactose standards. Spikes of 0.10 M lactose were added to the reaction mixture containing 200 µL of lactose, 50 µL of PABA, and 10 mg of sodium cyanoborohydride. The results of this study are shown in Table 11.
Table 9. Results of Recovery Studies of Lactose Spikes Added to Carnation Dry Milk

<table>
<thead>
<tr>
<th>Lactose spike volume (µL)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>25</td>
<td>111.7</td>
</tr>
<tr>
<td>50</td>
<td>88.5</td>
</tr>
<tr>
<td>30</td>
<td>123.4</td>
</tr>
<tr>
<td>60</td>
<td>103.2</td>
</tr>
</tbody>
</table>

Note: The lactose spike was added to the reaction mixture containing Carnation non-fat dry milk. The reaction was prepared and injected in triplicates. The reaction was carried out at 60°C for 45 minutes.

Table 10. Recovery Results for Nestle Good Start infant formula

<table>
<thead>
<tr>
<th>Lactose spike (µl)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>98.9</td>
</tr>
<tr>
<td>100</td>
<td>102.6</td>
</tr>
</tbody>
</table>

Note: Lactose spikes were added to the reaction carried out at 60°C for 45 minutes. Each reaction was prepared and injected in triplicates.
Table 11. Recovery Result for 0.010 M Lactose Standard

<table>
<thead>
<tr>
<th>Lactose spike (µL)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>98.8</td>
</tr>
<tr>
<td>30</td>
<td>130.1</td>
</tr>
</tbody>
</table>

Note: Lactose spikes were added to the reaction carried out at 60°C for 45 minutes. Each reaction was prepared and injected in triplicates.

The overall average, recovery for all samples tested, was 107.1%. This indicates that the method is quite accurate for the determination of lactose in commercial samples. The recovery is within 10% of the expected results from the calibration curve. The recovery range is 88.5 – 130.1 with a relative standard deviation of 12.9%. This is an acceptable level of precision. The variation can be explained by the multiple steps required in the analysis process including the derivatization procedure and the final analytical method. It can further be explained by the variation in the samples tested, which had varied sample matrices, including a powdered beverage, a liquid infant formula, and a standard lactose solution.

Comparison of the HPLC Method to Alkaline Ferricyanide Method

To further confirm the validity of the developed HPLC method, results were compared to a more traditional and accepted method, the alkaline ferricyanide method for UV detection. For this method 1-3 µg of lactose was mixed with 1 mL of the alkaline cyanide and 1 mL of ferricyanide solution. The mixture was heated for 15 minutes in a boiling water bath. Upon cooling, 5 mL of ferric ammonium sulfate solution was added. The resulting solution’s absorbance was measured at 700 nm using a Milton Roy Spectronic 301 spectrophotometer. A calibration curve was constructed consisting of
1.78 - 8.9 µg of lactose from the standard lactose solution. Results for Gerber Banana Smoothie juice, Carnation non-fat milk, Nestle Good Start formula, and Gerber Graduates Berry Smoothie juice were obtained and compared to the HPLC method. These results were further compared to the manufacturer’s reported concentrations of lactose per serving.

The alkaline ferricyanide method is a classical method for the determination of reducing sugars. Upon heating, reducing sugars will react with alkaline ferricyanide above pH 10.5 to form a ferrocyanide derivative that further reacts with ferric salts, ferric ammonium sulfate in this instance, to form Prussian blue. The colored derivative can then be determined by colorimetric detection or spectrophotometrically.

Three regression plots were generated, HPLC method versus UV method, HPLC vs. Manufacturer’s reported values, and UV versus manufacturers reported values. All three plots show a strong linear relationship. These results indicate that all three results are comparable, once again validating the developed method of analysis of lactose by HPLC. The correlation coefficient, R, for the HPLC method compared to the alkaline ferricyanide method was 0.99. The correlation coefficient, R, for the HPLC method compared to the manufacturers’ results was 0.999. The slopes for the regression lines of the HPLC method versus the alkaline ferricyanide method and the HPLC method versus the manufacturers’ reported concentrations were 0.88 and 0.71, respectively. This suggests that the results between the two analytical methods agree more strongly with each other than the manufacturers’ results.

Most of the variation between the HPLC method and the manufacturers’ values can be explained by the juice samples. The greatest difference between the analytical
methods and the manufacturers’ reported lactose is observed within the Gerber smoothie juices. The alkaline ferricyanide method shows better agreement with the manufacturer than with the HPLC method for these samples. This is most likely due to an interfering species within the sample matrix that also reacts with PABA to produce a UV absorbing product.

The manufacturers’ reported content of lactose seems somewhat different than the lactose determined by either the HPLC or the ferricyanide method. This can be explained by the variation within the manufacturing process. The amount of lactose reported on the nutritional label represents the average lactose in the product throughout various batches. Therefore, there is a potentially significant margin of error in the reported lactose on the nutritional label compared to the actual lactose concentration per serving.

The regression plots further show that the HPLC method is accurate when compared to the alkaline ferricyanide UV method. There is a strong correlation between the results with correlation coefficients nearly equal to one. The correlation coefficient for comparing the HPLC method and the manufacturers reported lactose is slightly closer to one than the UV method compared to the manufacturers’ reported lactose. This indicates that the HPLC method agrees more closely to the label claims regarding lactose per serving. These results coupled with the recovery studies, 107% average recovery, agree and establish that the HPLC method can be used to accurately determine the lactose content of commercial food samples.
Table 12. Comparison of Lactose Content as Determined by HPLC and Alkaline Ferricyanide Method

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPLC method (mg/ml)</th>
<th>Alkaline ferricyanide method (mg/ml)</th>
<th>Manufacturer’s report (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestle Good Start</td>
<td>57.00</td>
<td>60.38</td>
<td>74.67</td>
</tr>
<tr>
<td>Carnation dry milk</td>
<td>42.72</td>
<td>37.4</td>
<td>50</td>
</tr>
<tr>
<td>Gerber Banana Smoothie</td>
<td>7.75</td>
<td>2.46</td>
<td>2.1</td>
</tr>
<tr>
<td>Gerber Berry Smoothie</td>
<td>7</td>
<td>2.46</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Figure 19. Plot of lactose concentration obtained by the HPLC method versus the lactose concentration obtained by the UV method. The equation of the regression line is $y = 0.883x + 5.9466$ with a correlation coefficient, $R$, of 0.990.

Figure 20. Plot of lactose concentration determined by the HPLC method versus the label reported lactose concentration per serving. The equation of the regression line is $y = 0.7062x + 5.5048$ with a correlation coefficient, $R$, of 0.999.
Figure 21. Plot of lactose concentration determined by the UV method compared to the manufacturers’ label concentration. The equation of the regression line is \( y = 0.7954x - 0.3574 \) with a correlation coefficient of 0.998.
CHAPTER 5
CONCLUSIONS

Lactose intolerance affects many people world wide. Its symptoms can be mild or severe depending upon the level of lactase deficiency. Because small amounts of lactose can cause large amounts of discomfort and illness, it is important that a sensitive and reliable method be available for the determination of lactose in food samples. This is especially important for infant foods because milk is the exclusive energy source during the first part of life.

HPLC is a common technique for analysis; however, for the determination of carbohydrates refractive index detection is most often employed since carbohydrates only absorb in the lower wavelength region, 190-210nm. Refractive index, while readily available, suffers from the lack of selectivity and sensitivity. Conditions under which samples are analyzed must be carefully controlled. Temperature, pressure, and purity are of critical importance because they can lead to inaccurate results.

During this project, a sensitive, reliable, and selective method for the determination of lactose was developed and applied to commercial products. The pre-column derivatization by reductive amination with p-aminobenzoic acid allowed for detection by a UV diode array detector. The detection wavelength region of 300-360nm reduced interference by other organic species. The derivativization reaction itself was quite simple to perform and required relatively inexpensive reagents. The conditions under which the reaction proceeded were mild and easily controlled. This is a strong advantage when compared to enzymatic reactions were reagents are more expensive and the reaction conditions are much more delicate. The reaction offered a strong advantage
in being selective to reducing and aldose form sugars. There was no interference from sucrose or fructose, other common sugars occurring in food samples.

The separation of glucose and lactose derivatives was accomplished using a common C8 column and an 85% methanol and 15% deionized water mobile phase. The C8 column is readily available in most analytical laboratories and is much less expensive than a specialty column. The average cost of a C8 column is approximately $300 compared to approximately $1000 for some carbohydrate columns, which may also require more specialized and expensive mobile phases. The mobile phase consisting of methanol and deionized water is of course very inexpensive. It also offers the advantages of being benign and environmentally friendly, requiring no special disposal procedures.

The method was consistently reproducible throughout the course of experimentation. During reproducibility studies, the relative standard deviation of results for lactose was 4.96%. Within a mixed sample containing both glucose and lactose reproducibility suffered slightly with the relative standard deviations for glucose and lactose being 13.29% and 6.48% respectively. These are still quite acceptable results.

The method also showed a linear relationship between peak area and concentration. The correlation coefficient, R, was determined to be 0.96, indicating a strong linear dependency of peak area on lactose concentration. The lower limit of detection was the point where the relationship was no longer linear, and the results became considerably less reproducible. For this method, the lower limit of detection for lactose was 0.0036 mg/ml. This is quite an acceptable level for application to food samples.
The accuracy of the method was also studied carefully by recovery studies and comparison to the ferricyanide UV method and the manufacturers’ reported lactose amounts per serving of food sample tested. The average recovery of lactose was 107%. The correlation between the HPLC method with the traditional ferricyanide method was quite strong as was the HPLC method with the manufacturers’ reported lactose levels. When the results were plotted, correlation coefficients were nearly 1.

Finally, the HPLC method was applied to commercial food samples. The level of lactose was accurately determined in infant formulas, infant juice drinks, and non-fat dry milk. Very little sample preparation was needed. The only step prior to the derivatization reaction was the extraction of fat using ethyl ether.

The developed method for the determination of lactose by reversed-phase HPLC shows all of the desired characteristics, sensitivity, selectivity, and reproducibility. The use of UV detection was accomplished with a simple derivatization procedure requiring mild conditions and inexpensive reagents. The separation was achieved with low cost stationary and mobile phase components that are environmentally friendly and readily available. And most importantly the technique was applied for the accurate quantitation of lactose in commercial samples.
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