3. QUANTITATIVE ANALYSIS OF CARBOHYDRATES

3.1 Milk and its properties

Milk is a fluid secreted by the mammary glands. The composition of milk contains a large amount of water and many solids. Emulsified lipids and calcium salts of casein give milk a white appearance while carotenoid pigments make the milk yellow.

Casein, lactalbumin and lactoglobulins are the proteins in the milk. Casein is a phosphoprotein and usually find in the form of calcium caseinate. In case of acid effect and fermentation, casein and calcium are separated from calcium caseinate, casein precipitate together with oil. Palmitic, oleic, stearic, myristic acids and triglycerides of high fatty acids are abundant in milk. Vitamins of A, D, E, K and B_2 vitamin and catalase, peroxidase, phosphatases are also the content of the milk.

Composition of milk can vary by environment; however common content of cow milk can be listed as it is given in the following table:

Compounds	Percentage
Water	87.3%
Protein	0.75%
Mineral	3.40%
Lactose	4.70%
Fat	3.50%
Vitamin	0.35%

Lactose is carbohydrate found in milk. It is a reducing disaccharide consisting of glucose and galactose monosaccharides (**Figure 3.1**). The digestion of lactose in our body breaks down through the lactase enzyme (β-galactosidase), which is secreted from small intestinal wall cells. Glucose and galactose monosaccharides formed as a result of hydrolysis of lactose with this enzyme are absorbed by the intestine and pass through the bloodstream and metabolized in the liver.

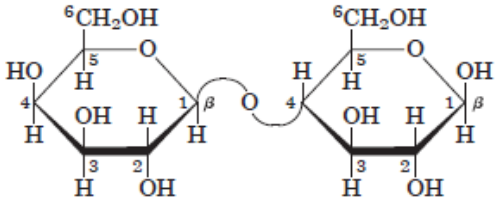

Figure 3.1 Formula of β-lactose

Lactose intolerance is mentioned when the lactase enzyme is deficient or not work properly**.** Lactose intolerance can be defined as the situation that one is not able to digest milk and its products. This condition called milk intolerance or lactase deficiency is one of the most common digestive disorders in the world and is more common especially in Asian-European races. The symptoms of lactose intolerance are excessive gas, nausea, and diarrhea. If there is no lactase activity in the intestines or if it is low, the undigested lactose disrupts the osmotic balance and causes fluid and electrolyte accumulation in the intestine. In expanding intestines, mobility increases and diarrhea occurs. In addition, undigested lactose is fermented by the bacteria in the intestine and hydrogen gas is produced. Excessive amounts of hydrogen both increase diarrhea and cause other digestive system disorders, especially gas and bloating. These symptoms appear 30-120 minutes after taking lactose-containing foods.

Although lactose intolerance is an annoying disorder, it is not fatal. Lactose intolerance is diagnosed if abdominal pain and complaints occur, after avoiding foods containing lactose for several days and consecutively drinking 2-3 glasses of water. It is accurately diagnosed by clinical tests:

a) Lactose tolerance test: After fasting blood glucose is recorded, lactose is given to patient and then blood glucose is measured several times. Increasement in blood sugar level means that there is no lactose intolerance.

(b) Breath test: After intake of lactose, hydrogen gas is measured in the breath.

(c) Biopsy: It is conducted biopsy on intestines.

The most effective way for treatment is to decrease or remove lactose-containing products from the diet with respect to severity. While to decrease the amount of dairy products is enough for the treatment if complaints are mild, a severe lactose-free diet may be required for severe cases.

In very sensitive people, even a small amount of lactose in the coffee cream can cause complaints.

The most frequently consumed lactose sources are; milk, butter, margarine, yogurt, cheese, milk powder, some types of bread, dough products and chocolate.

Yogurt is traditionally consumed throughout the world among populations who are seemingly unable to digest lactose. The lactose in yogurt is digested more efficiently than other dairy sources of lactose because the bacteria inherent in yogurt assist with its digestion.

3.2 Blood Glucose

In a healthy person, fasting blood sugar is 80-120 mg / 100 mL (glycemia). After meal, glucose levels increase. The 2-hour postprandial blood sugar is 140 mg / 100 mL. Blood glucose level can fall below normal value (hypoglycaemia) as well as rising (hyperglycemia). Hyperglycemia (diabetes, Diabetes Mellitus) is a hormonal disease, concerning protein and lipid metabolism, especially carbohydrate.

There are hormones that regulate the blood glucose levels in our body. Insulin, which prevents blood glucose level rising, and glucagon, which prevents blood glucose level dropping, are the most important among of them. The pancreas, which secretes these two hormones, and the liver working as a glucose storage, are the most important organs in blood sugar level adjustment. The liver collects and stores glucose from blood in response to the message bring by insulin as a result of that blood sugar drops. Glucagon transfers glucose stored by the liver to the bloodstream and raises blood glucose level.

The main problem in diabetes mellitus patient is that glucose in the bloodstream cannot enter the cells. Glucose, which we normally take with nutrients or released into the blood from liver, enters the cells from the blood and is converted into energy by the binding of the insulin hormone secreted by the beta cells of the pancreas to a receptor present in the cell membrane. Diabetes is a disease that develops due to the lack of or ineffective action of insulin.

There are two major clinical classes of diabetes mellitus: type I diabetes, or insulin dependent diabetes mellitus, and type II diabetes, or non-insulin-dependent diabetes mellitus, also called insulin-resistant diabetes.

Type 1 Diabetes: This type of diabetes, also called insulin-requiring diabetes, is more common in children and young adults. The pancreas produces little or no insulin. Since there is not enough insulin in the blood to fill insulin receptors, glucose cannot get into the cells. Patients must take life-

long insulin hormone (by injecting) as it is an absolute or relative insulin deficiency. They make up 8-10% of all diabetes patients in the community.

Type 2 Diabetes: This type of diabetes, also called insulin-free diabetes, is frequently seen in adults and obese people. In type 2 diabetes, insulin secretions are normal and there are problems with cell receptors. Glucose cannot enter cell because insulin cannot bind to cell receptors. In this case, glucose is continuously drawn from the glycogen stores and blood sugar rises to meet the energy needs of the cell. It is the most common type of diabetes. It accounts for about 90% of all diabetics.

Symptoms of diabetes

Diabetes is related to the abnormalities in carbohydrate, fat, and protein metabolism in the body. For whatever reason, negative effects of excess glucose in the blood are seen. When glucose rises above the blood serum level (180 mg / 100 mL blood), it begins to be excreted in the urine. Glucose excreted by the kidneys also increases fluid excretion, result in excessive urination (polyuria). The body tries to meet the liquid it loses with polyuria by drinking large amounts of liquid (polydipsia). Since the organism cannot use glucose as an energy source, appetite increases and starts to use backup energy stores. When the body uses lipids to get energy, molecules called ketone bodies produces. When ketone bodies accumulate, ketoacidosis occurs. Depending on the accumulation of acetoacetic acid and beta-hydroxybutyric acid, acidosis (decrease in HCO^{-}_{3} level in plasma) occurs.

3.3 Experiments

3.1 Determination of lactose content of milk by Benedict's Method

Principle of the Experiment: Benedict reagent for the estimation of reducing sugars contains potassium thiocyanate as well as copper sulfate, and in the presence of the former a white precipitate of cuprous thiocynate is formed in the reduction instead of the usual red precipitate of cuprous oxide.

Laktose + $Cu^{+2} \rightarrow$ CuOH + β-D-Glucose + β-D-Galactose

CuOH + KSCN $\rightarrow$ KOH + CuSCN $\downarrow$

Procedure:

1st Step: Precipitation of Casein in Milk (Protein free milk solution)

(1) Add 2 mL milk, 1 mL 10% sodium tungstate and 1 mL 0.7 N H_2SO_4 to test tube and wait 5 minutes.

(2) Add 16 mL water to the solution prepared in step complete the final volume to 20 mL.

(3) Filter the solution until it becomes transparent clear liquid, separate the precipitate load the burette with filtrate.

2nd Step: Quantitative Analysis of Lactose (Lactose Content of the Milk)

(1) Pour 5 mL quantitative Benedict's reagent to an erlenmeyer flask and add trace amount of Na_2CO_3.

(2) Heat the solution until Na_2CO_3 is dissolved completely. When the solution starts to boil, the filtrate in the burette is added dropwise (During titration, shake and boil the solution continuously).

(3) Continue to titrate until white precipitation occurs. Note how much filtrate is used from the burette for titration.

In this experiment;

Na_2CO_3 makes the medium alkaline.

Sodium citrate prevents the precipitation of CuOH in solution.

Ferrocyanide prevents the precipitation of reduction product copper oxide in solution.

Potassium thiocyanate reacts with the Cu^+ ions and used as titrant.

Calculation: 13.4 mg of lactose required to titrate 5.0 mL of Benedict's solution

%g laktoz = [13.4x100x dilution factor]/ [the volume of filtrate consumed during titration x 1000]

Reagents

Benedict's quantitative solution:

A solution: Dissolve 10 g anhydrous Na_2CO_3, 20 g sodium citrate and 12.5 g potassium thiocyanide in 60 mL water while heating. Cool it to room temperature and filtrate it.

B solution: Dissolve 1.8 g copper sulphate in 10 mL pure water. Add 5.0 mL of 5.0% potassium ferrocyanide solution.

Mix A and B solutions and dilute it to 100 mL with distilled water.

Sodium tungstate: (10.0% w/v solution) 10 g sodium tungstate is dissolved in 100 mL distilled water.

Sulfuric acid (0.7 N H_2SO_4): Add 19 mL concentrated H_2SO_4 to 400-500 mL water and dilute it to 1 L.

Experiment 3.3.2 Spectrophotometric Lactose Analysis in Milk (Protein free milk solution)

Procedure:

(1) Take 250 μL of milk and add 500 μL of 10% Na-tungstate solution. Then add slowly 500 μL of 0.75 N sulfuric acid solution while stirring it slowly. Mix well and complete the volume of the solution to 25 ml with water stand for 5.0 min. Mix well and filter.

(2) Prepare series of tubes as shown in the following table

Solutions /mL	Sample	Standard	Blank
Filtrate	0.5	-	-
Lactose standard	-	1	-
Distilled water	0.5	-	1
Cupper reagent	1	1	1

(3) Tubes are heated gently in water bath at 8 min. Cool and add 2 mL phosphomolybdic acid and wait 10 min (during completed gas outlet)

(4) Read the absorbance at 630 nm against reagent blank.

(5) Calculate the amount of lactose in milk with the following formula.

Calculation:

% Lactose = [Absorption of sample / Absorption of standart]

Reagents:

0.7 N H_2SO_4 solution: 1.9 mL concentrated H_2SO_4 is added to 40-50 mL distilled water and dilute it to 100 mL.

Copper reagent: Dissolve 40 g sodium carbonate (Na_2CO_3) in 400 mL distilled water. Add and mix 7.5 g tartaric acid and 4.5 g $CuSO_4.5H_2O$. Dilute final volume to 1 L.

0.2% benzoic acid solution: Dissolve 0.2 g benzoic acid in 100 mL distilled water, boil and cool.

1% lactose solution: Dissolve 1 g lactose in 100 mL distilled water.

Standard lactose solution: 1.5 mL 1% lactose solution / 50 mL 0.2 % benzoic acid.

10% NaOH solution: 10 g NaOH/ 100 mL distilled water.

Phopshomolybdic Acid : Boil the 3.5 g molybdic acid, 0.5 g sodium tungstate and 20 mL 10% NaOH solution until all the ammonia is released in molybdic acid approximately 20-40 minutes. Add 12.5 mL 85%phosphoric acid and dilute the final volume to 50 mL with distilled water.

Experiment 3.3. Determination of Glucose in Blood (Folin & Wu's method)

Principle of the Experiment: Glucose and other reducing substances under alkaline conditions reduce the cupric ions of the copper reagent to cuprous ions. Cuprous oxide reacts with phosphomolybdic acid to from molybdenum blue. Intensity of molybdenum blue is directly proportional to the reducing sugars present. Absorption is measured at 420 nm and concentration of glucose is obtained from calibration curve.

$2Cu^{+2}$+ Glucose $\rightarrow$ Gluconic acid + $Cu_2O \downarrow$ (red)

$2Cu^{+}$+ $Mo^{+4} \rightarrow Mo^{+2}$+ $2Cu^{+2}$ (blue)

Procedure:

1st Step: Deproteinization of blood

Solutions / mL	**Blank**	**Sample**
Distilled water	4	3.5
Serum	-	0.5
0.7 N H_2SO_4	0.5	0.5
10% sodium tungstate	0.5	0.5
Shake the tubes, wait them at room temperature 5 minutes and filtrate		

2nd Step: Reduction and color development

Take 2 Folin-Wu tubes and add solutions which are given table. **Top of the each tube should be covered with aluminum foil during heating and incubation processes (Discuss why Folin-Wu tubes are used in this experiment).**

Solutions (mL)	Blank	Sample
Blank filtrate	1	-
Sample filtrate	-	1
Copper reagent	1	1
Mix and keep in boiling water bath for 8 minutes and cool		
Phosphomolybdic acid	1	1
Mix and keep in boiling water bath for 5 minutes and cool under running tap water		
Distilled water	9.5	9.5
Keep in room temperature for 10 minutes		
Measure the optical density of samples at 420 nm by using the blank solutions		

Standard calibration curve of glucose

(1) Take 4 Folin-Wu tubes and add solutions which are given table. **Top of each tube should be covered with aluminum foil.**

Solutions/ mL	1	2	3	4
Std glucose solution	**0.25**	**0.50**	**0.75**	**1**
Distiled water	0.75	0.50	0.25	-
Copper reagent	1	1	1	1
Mix and keep in boiling water bath for 8 minutes and cool under running tap water				
Phosphomolybdic acid	1	1	1	1
Mix and keep in boiling water bath for 5 minutes				
Distilled water	9.5	9.5	9.5	9.5

Keep in room temperature for 10 minutes

Read the colour of standards at 420 nm

Find the concentration of glucose (mg of glucose/100 mL blood) in sample as mg% in sample from the calibration curve.

Reagents

0.25% benzoic acid solution: Dissolve 0.625 g benzoic acid in 250 mL distilled water, boil and cool. Compensate the lost solution due to vaporization by distilled water.

Stock glucose solution (10 mg/mL): Dissolve 1 g anhydrous glucose in 5 mL 0.25% benzoic acid and dilute it to 100 mL.

Standard glucose solution (0.2 mg/mL): Add 2 mL of glucose stock solution onto the 50 mL 0.25% benzoic acid solution, then mix the solution. Dilute it to 100 mL with benzoic acid solution.

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