Date ..... Name ..... Group .....

Instructions for the practical lesson on biochemistry

Topic: **Reactions of saccharides. Thin layer chromatography. Polarimetry**

Task 1: Analysis of unknown sample of saccharide by means of color reactions

Tested samples:

Glucose	10 g/l
Fructose	10 g/l
Xylose	10 g/l
Maltose	10 g/l
Sucrose	10 g/l
Starch	10 g/l

Unknown samples of saccharides

Molisch Reaction

Reagents:

Molisch reagent: solution of *α-naphthol 100 g/l in 96 % ethanol* 

Concentrated sulfuric acid 

Procedure:
Prepare the reaction mixtures in test tubes according to the following table:

	Test tube 1 FRUCTOSE	Test tube 2 MALTOSE	Test tube 3 UNKNOWN	Test tube 4 BLANK
Fructose	cca 1 ml	-	-	-
Maltose	-	cca 1 ml	-	-
Unknown sample	-	-	cca 1 ml	-
Distilled water	-	-	-	cca 1 ml
Molisch reagent	cca 2 drops	cca 2 drops	cca 2 drops	cca 2 drops
Shake briefly with the mixture in each tube. Then carefully underlie each mixture with concentrated sulfuric acid. In the presence of saccharide a violet ring forms in the interface.				

Bial reaction

Reagents:

Bial reagent: *0.3 g orcinol in 100 ml 30 % HCl and 5 drops $FeCl_3$ 100 g/l* 

Procedure:

	Test tube 1 XYLOSE	Test tube 2 GLUCOSE	Test tube 3 UNKNOWN	Test tube 4 BLANK
Xylose	cca 5 drops	-	-	-
Glucose	-	cca 5 drops	-	-
Unknown sample	-	-	cca 5 drops	-
Distilled water	-	-	-	cca 5 drops
Bial reagent	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml
Place the tubes to a hot water bath and keep observing the color changes. Read the results after 2-3 minutes.				

Selivanov reaction

Reagents:

Selivanov reagent: *Solution of resorcinol 5 g/l in HCl 200 g/l* 

Procedure:

	Test tube 1 GLUCOSE	Test tube 2 FRUCTOSE	Test tube 3 SUCROSE	Test tube 4 UNKNOWN	Test tube 5 BLANK
Glucose	cca 0.5 ml	-	-	-	-
Fructose	-	cca 0.5 ml	-	-	-
Sucrose	-	-	cca 0.5 ml	-	-
Unknown sample	-			cca 0.5 ml	-
Distilled water	-	-	-	-	cca 0.5 ml
Selivanov reagent	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml	cca 1.5 ml
Place the tubes to a boiling water bath and keep observing the color changes. Read the results after 1-2 minutes.					

Benedict reaction

Reagents:

Benedict reagent:

$CuSO_4.5\ H_2O$ 17.3 g is dissolved in about 100 ml of distilled water.
Na_2CO_3 100 g & sodium citrate 173 g is dissolved in about 700 ml of distilled water.
Both solutions are combined and the volume is adjusted to 1 L with distilled water.

$CuSO_4$ 

Procedure:

	Test tube 1 GLUCOSE	Test tube 2 MALTOSE	Test tube 3 SUCROSE	Test tube 4 ASCORBIC ACID	Test tube 5 UNKNOWN	Test tube 6 BLANK
Benedict reagent	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Glucose	cca 4 drops	-	-	-	-	-
Maltose	-	cca 4 drops	-	-	-	-
Sucrose	-	-	cca 4 drops	-	-	
Ascorbic acid	-	-	-	cca 4 drops	-	-
Unknown sample	-	-	-	-	cca 4 drops	-
Distilled water	-	-	-	-	-	cca 4 drops
Shake briefly with the mixture in each tube and place the tubes to a boiling water bath. Read the result after 3-4 minutes. Evaluate both color change and occurrence of precipitate.						

Barfoed reaction

Reagents:

Barfoed reagent:

13.3 g of neutral crystalline copper acetate is dissolved in 200 ml of distilled water, filtered and 1.8 ml of glacial acetic acid is added

Glacial acetic acid  *Copper acetate* 

Procedure:

	Test tube 1 GLUCOSE	Test tube 2 MALTOSE	Test tube 3 SUCROSE	Test tube 4 UNKNOWN	Test tube 5 BLANK
Glucose	cca 0.5 ml	-	-	-	-
Maltose	-	cca 0.5 ml	-	-	-
Sucrose	-	-	cca 0.5 ml	-	-
Unknown sample	-	-	-	cca 0.5 ml	-
Distilled water	-	-	-	-	cca 0.5 ml
Barfoed reagent	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Place the tubes to a boiling water bath. Read the result after 2-3 minutes.					

Reaction with the Schiff reagent

Reagents:

Formaldehyde 5 g/l 

Schiff reagent: Fuchsine solution decolorized with sodium hydrogen sulfite 

Procedure:

	Test tube 1 GLUCOSE	Test tube 2 FORMALDEHYDE	Test tube 3 UNKNOWN	Test tube 4 BLANK
Glucose	cca 1 ml	-	-	-
Formaldehyde	-	cca 1 ml	-	-
Unknown sample	-	-	cca 1 ml	-
Distilled water	-	-	-	cca 1 ml
Schiff reagent	cca 2 drops	cca 2 drops	cca 2 drops	cca 2 drops
Shake briefly with the mixture in each tube and read the result.				

Reaction for demonstration of starch

Reagents:

Lugol solution: *solution of iodine 3 g/l in solution of potassium iodide 50 g/l*

Procedure:

	Test tube 1 GLUCOSE	Test tube 2 STARCH	Test tube 3 UNKNOWN	Test tube 4 BLANK
Glucose	cca 1 ml	-	-	-
Starch	-	cca 1 ml	-	-
Unknown sample	-	-	cca 1 ml	-
Distilled water	-	-	-	cca 1 ml
Lugol solution	cca 2 drops	cca 2 drops	cca 2 drops	cca 2 drops
Shake briefly with the mixture in each tube and read the result.				

Transfer all the relevant results of the color tests for the unknown sample into the scheme in your lab report form. Based on the analysis you have done, try to characterize the unknown sample as closely as you can, e.g. whether it appears to be a reducing or non-reducing saccharide, hexose or pentose, and so on.

Task 2: Thin layer chromatography of saccharides

Reagents:

1. Standards: aqueous solutions of galactose, maltose, lactose, and fructose 10 g/l
2. Unknown sample of saccharide
3. Ethyl acetate 
4. Isopropanol 
5. Diphenylamine 
6. Aniline 
7. Phosphoric acid 85 % 
8. Acetone 

Preparation of experiment (performed by technician):
Detection reagent: 4 g of diphenylamine, 4 ml of aniline and 20 ml of 85 % phosphoric acid is dissolved in 200 ml of acetone.
Elution system: to a chromatographic chamber measure 15 ml of ethyl acetate, 12 ml of isopropanol and 3 ml of distilled water, close and mix. Let the chamber saturate with solvent vapors for about 15 minutes. Work in a functioning fume chamber.

Procedure:

1. Preparation of the sheet and application of samples
On a Silufol sheet of width 7.5 cm mark with ordinary pencil (not ball pen!) five start positions about 1.5 cm from the lower edge. The distance between the starts should be about 1.5 cm. Write some codes of the applied substances on the opposite margin of the sheet. Apply about 3-4 µl of the solutions of galactose, maltose, lactose, and fructose to four of the marked starts, respectively, and the unknown sample to the fifth position. The start spots should not be larger than 0.5 cm. Allow the starts to dry completely.

2. Development
Carefully place the sheet to the chromatographic chamber with prepared elution system and close the chamber immediately. The starts themselves must not be immersed. Terminate the chromatographic separation when the solvent reaches about 2-3 cm from the upper edge. Dry the sheet in the oven set to 85 °C.

3. Detection
Spray the dry chromatogram with the detection reagent and place again to the pre-heated oven for 2-3 minutes. After heating colored spots of saccharides appear. Detection is performed in functioning fume chamber (!) **No open flame is allowed in the laboratory during this procedure!**

Task 3: Inversion of sucrose

Reagents:

a) Sucrose solution of unknown concentration

b) Concentrated HCl 

Procedure:

1. Measurement of the optical activity of sucrose solution
Fill the polarimetric cuvette with the solution of sucrose and measure its optical activity α (refer to the instructions at the polarimeter for detail). Empty the polarimetric cuvette after measurement and rinse it well with distilled water.

2. Hydrolysis of sucrose
Measure 30 ml of the sucrose solution to a beaker and add 5 drops of concentrated hydrochloric acid. Mark the level of the solution on the beaker. Heat the acidified sucrose solution in a water bath for 10 minutes and put aside to cool. Add distilled water to replenish the evaporated liquid up to the mark and mix.

3. Measurement of the optical activity of hydrolysate of sucrose
Fill the polarimetric cuvette with the cooled solution of hydrolyzed sucrose and measure its optical activity α. Empty the polarimetric cuvette after measurement and rinse it well with distilled water.

Evaluation:

1. Calculation of the original concentration of sucrose:

$$w\,(g/l) = \frac{\alpha \times 100}{[\alpha]_D^{20°C} \times 1}$$

Length of cuvette l = 0.2 m

Specific rotation for sucrose:
$[\alpha]_D^{20°C} = +66.5\,°$

2. Calculation of the optical activity of invert sugar after hydrolysis of sucrose (verification of hydrolysis completion):

The complete hydrolysis of sucrose produces an equimolar mixture of glucose and fructose, with concentration of each monosaccharide being equal to the concentration of sucrose before hydrolysis. If we know the original sucrose concentration, and also the specific activity values for glucose and fructose, we can predict the optical activity of the solution after hydrolysis.

First convert the value of the original sucrose concentration (g/l) to mol/l. It gives you the predicted concentration of the hydrolysis products glucose and fructose. Because the specific activity concentration is defined in terms of mass concentration, not molar, convert the predicted glucose and fructose concentrations (w) to g/l[1].

Next, calculate the predicted optical activity of the solution after hydrolysis using the formula:

$$\alpha = [\alpha]_{D\ \text{D-glucose}}^{20°C} \times \frac{l \times w}{100} + [\alpha]_{D\ \text{D-fructose}}^{20°C} \times \frac{l \times w}{100}$$

Specific activity for D-glucose: $[\alpha]_D^{20°C} = +52.5\,°$; for D-fructose: $[\alpha]_D^{20°C} = -92.4\,°$

Finally, compare your measured value of the optical activity after hydrolysis of sucrose with the predicted one. If they match, it is evidence for the complete hydrolysis. If the measured value is higher (less negative) it indicates that there is still significant residual sucrose in the solution.

[1] Note that it is not adequate to simply halve the sucrose concentration in g/l because molecules of water enter the hydrolytic reaction and become incorporated into the hydrolysis products.