PRACTICAL 5: ELECTROPHORESIS AND THIN LAYER CHROMATOGRAPHY

Aims and Learning Objectives:

1. To separate amino acids (charged molecules) by paper electrophoresis.

2. To identify amino acids in a mixture following electrophoretic separation and ninhydrin staining.

3. Use of a TLC technique and aniline diphenylamine to separate and identify sugars respectively in a mixture.

4. To distinguish between aldose and ketose sugars, and mono- and disaccharides, by reaction with aniline diphenylamine to give a variety of coloured products.

Class assessment: 5% of module mark

1. Practical 5 Worksheet (100%)

A. ELECTROPHORESIS

Background

Electrophoresis is defined as the movement of charged molecules in solution under the influence of an electrical field. The rate of migration varies for different ions and this difference is used to separate components in a mixture e.g. serum proteins, nucleic acid.

The migration of proteins or amino acids in an electrical current depends mainly on three factors:

(a) Nature of the charged protein e.g. size and sign (+ or -) of its net charge.

(b) Buffer solution e.g. pH and ionic strength (conductivity).

(c) Electrical field e.g. intensity and distribution along the migration support medium.

The direction of migration is towards the electrode of opposite charge. The pH of the buffer that provides the environment for the protein usually determines the net or overall charge on the molecule, especially in the case of an amino acid or protein that carries both positive and negative charges e.g. at **acidic pH** an **amino acid** will act as a **cation** whilst at **alkaline pH** conditions, behave as an **anion**. At the **isoelectric point (pI)** the net charge is zero and no migration occurs. Furthermore, at ***pH*** values ***below*** the ***pI***, ***proteins*** behave as ***positively*** charged ***cations*** whilst at pH values ***above*** the ***pI***, proteins behave as ***negatively*** charged ***anions***.

Due to the passage of current through the paper there will be a **heating effect** and water will evaporate from the paper but since the ends are dipped in tanks of buffer, more will be sucked into the paper by capillarity. Increasing the ionic strength of the buffer causes decreased ion mobility and the increased conductivity causes an undesirable increase in the heating effect.

Two other complications are diffusion and electro-osmosis. **Diffusion** of the applied material, which is of course time-dependent, is minimised by using small areas of application of the sample and also by using the shortest possible time for the experiment with highest permissible voltages. It is also best to use the smallest detectable amounts of material to avoid overloading of the paper. **Electro-osmosis** is due to the fact that water becomes positively charged relative to filter paper when the two are in contact and when the potential difference is applied, buffer streams towards the cathode carrying with it all dissolved substances. This effect can be followed by adding a neutral compound, eg, glucose and after the experiment, the position of this marks the position where molecules at their iso-electric points should be found, ie. the true origin. Electro-osmosis is minimised in vertical electrophoresis where the paper is arranged so that the flow of buffer due to siphoning opposes that due to electro-osmosis.

In this practical, Whatman 1 chromatography paper will be used to separate amino acids by electrophoresis.

At a pH of around neutrality, amino acids such as **leucine** are electrically neutral, ie. they are at their isoelectric point. An acidic amino acid such as **aspartic acid** will however be negatively charged due to the ionisation of the side chain carboxyl group and a basic amino acid such as **lysine** will bear an overall positive charge. If these amino acids are placed on a filter paper soaked in 0.1M sodium ethanoate/ethanoic acid buffer at pH 5.5, application of an electrical current across the paper will cause lysine to move to the cathode, aspartic acid to the anode whilst leucine will remain almost at the original position.

Lab exercise I of 2

Avoid handling the paper with fingers –wear disposable gloves. Try not to tear the paper when damp.

1. Draw a pencil line carefully (avoid damage) across the middle of a 30 x 10 cm piece of the paper.

2. Mark on it with fine pencil four positions (2cm between each) corresponding to the standards leucine, lysine, aspartic acid and an "unknown" mixture that contains two of these amino acids.

3. Mark one end of the paper as positive (+) anode and the other negative (-) cathode.

4. Wet the paper all over with buffer (could place buffer in white tray for this purpose).

5. Remove excess buffer (avoid drying totally) by blotting with clean filter paper.

6. Assemble on the plastic stand in the tank ready for electrophoresis (see demo).

7. Fill the buffer tanks up to the level of the electrodes, keeping the levels equal to avoid siphoning. Make sure that no buffer spills into the middle tank.

8. Apply your samples to the four marked origin points on your drawn starting line. One small drop from a capillary tube is more than sufficient. N.B. Make sure that there is no

mixing of the amino acids between adjacent origin points.

9. Place the stand carefully into the tank, replace the lid and connect the electrodes but do not switch on. Make sure the polarity is correct. NOTE WHICH IS CATHODE AND ANODE!

10. The demonstrator will check the apparatus and switch on the MINI-300VS MINIS power pack which applies a voltage to the paper. Set the timer for 105 minutes, set the voltage to 300 V and finally press the on switch to start the run!

11. Condensation occurs on the sides of the tanks and this indicates that current is flowing through the paper. Allow to run for the 1¾ h.

12. The machine will bleep at the end of your run. Carefully remove the paper to dry it (for a few minutes).

13. Dip in ninhydrin stain and develop the colour using a hairdryer on full speed and maximum heat.

RESULTS

Measure the distance of movement on the amino acids from the origin line to centre of the spots noting towards which electrode migration has occurred. Put your measured distances together with direction of movement and likely charge on the amino acids on the results table. Put the names of your unknown amino acids (based on their movement/direction compared to the three standard amino acid markers) also on the table.

B. THIN LAYER CHROMATOGRAPHY

Background

One reagent that can be used for the detection of sugars, (such as may be present in a cell tissue / fluid or extract) is aniline diphenylamine. This reacts with sugars to produce a variety of

coloured end-products.

Commercially available TLC plates ("POLYGRAM SIL G"), comprising plastic sheets, pre-coated with silica gel powder as adsorbent, are used in this experiment. (The plates have already been cut to the correct size)

The one-dimensional technique involves applying small 'spot' samples at set intervals along a pre-drawn line on the TLC plate, and running the chromatograms in the usual way, using the chosen solvent system.

After drying, the plate is sprayed with aniline diphenylamine reagent, and the relative front (Rf) values of the sugars are then calculated, and compared to known 'marker' samples.

This experiment intends to establish the three main carbohydrate types present in an unknown fruit extract by use of one (single) dimension TLC.

Solutions of authentic sugar 'standards' (acting as markers), that might be expected as fruit constituents, will also be subjected to one-dimensional chromatography. This will enable the Rf values to be calculated, for the known sugars, in the solvent system being used.

These results will then be used to assist in the identification of the most common sugars present in the unknown extract sample.

Experimental

Safety spectacles, lab coats and protective disposable gloves must be worn at all times, throughout this experiment. Some of the chemicals being used are highly corrosive, and irritant.

In the event of any spillage of chemicals or breakage of equipment, please notify a member of staff immediately, so that the area can be safely decontaminated. Dispose of the glass 'spotters' in the special bins provided. Do NOT place them in the laboratory waste bins!

Lab exercise 2 of 2

1. Take one of the glass jars provided, remove the lid, and insert the filter paper along the inside wall, with the straight side on the base.
(This will act as a wick, and aid saturation of the atmosphere within the jar, by the solvent mixture).

2. Carefully pour 5 cm3 of the elution solvent, (1-butanol/ethanoic acid, 1:1 by volume) into the jar, and replace the lid. Leave the jar for at least 5 min, (to allow the solvent to saturate the airspace).

3. Take a 5cm. x 4.5cm. TLC plate, (handle carefully to avoid contamination and damage

to the silica gel coating), and, using a soft lead pencil, draw a line (origin line) across the bottom of the TLC plate, 4.5cm. long, and 1cm. from the end of the plate.

4. Starting 8mm. from the left hand side of the plate, mark on the origin line, a fine pencil dot or cross. Thereafter, make 3 more dots, each 10mm. apart.
(You should now have 4 dots on the origin line of your plate).

5. Repeat steps '3' and '4' above, on another TLC plate.

6. Apply *standard "marker" samples (see below) and the sample of fruit extract to the appropriate mark on the origin line of the plates.

7. *Standards to use as "markers" comprise both monosaccharides (aldohexoses - glucose, 2-deoxyglucose and galactose; ketohexose - fructose; pentose - ribose) and disaccharides (non-reducing sucrose and reducing maltose), all prepared in distilled water.

Suggestion - spot them onto the plate in the following order:

sucrose, glucose, 2-deoxyglucose and galactose (on one plate), and fructose, maltose, ribose and your fruit extract (on the other).

You are provided with a finely drawn capillary tube for 'spotting'.
Immerse the fine end of this tube in the sample of (sugar) solution for a few seconds, then withdraw it, and carefully apply ONE small spot of liquid to the TLC plate, on one of the marks on the origin line. Repeat for the remaining seven sugar solutions.

The 'spot' should not be more than 3mm. in diameter!

8. Allow the spotted TLC plates to dry for 5 min.

9. Remove the lid from the glass jar, and carefully place the two plates (with the origin lin at the bottom) into the jar, so that the top of the plates lean together, forming an inverted 'V' shape, with the silica layers facing inwards. (The solvent level should be such that it does not touch the sample spots).

10. Replace the lid, and leave the chromatogram to run for 20 min.
(You can use a timer for this).

11. At the end of the run time, remove the plates and carefully draw a pencil line across the plate (near the top) where the solvent has finally reached. (This is the Solvent Front). Allow the plates to dry in the air (approx. 15 min).

12. Location of Carbohydrates

Take your dried plates to a fume-cupboard, for spraying with the aniline diphenylamine

reagent, by a lab. demonstrator. The reagent is formulated to react with sugars in very low concentrations, and produces varied colours with different sugars.

Wearing protective gloves, place the sprayed plate in the fume-cupboard and use a hair-dryer to dry off the excess reagent. (Use the high heat setting and full fan speed on the dryer). Keep heating the plate until the coloured (sugar) spots develop.

RESULTS

Allow the plate to cool, then circle all the spots lightly, using a soft pencil, and note the colours produced as soon as possible. (The spots may begin to fade or change colour within a short time). Record the spot colours on the results table.

Measure the distance moved by each of the samples and put your values on the results table.

(Always measure from the centre of each spot to the origin line)

Calculate the retention values (Rf) for all of the spots on your chromatograms and put these values also on the results table.

(Show your Rf value working out in full in all cases for the full marks)

$$\mathbf{Rf} = \frac{\textbf{Distance from origin line to centre of coloured spot}}{\textbf{Distance of origin line to solvent front (line)}}$$

Based on the calculated Rf values, and the inspection of the coloured spots on your chromatograms, deduce which sugars are most likely to be present in your sample of fruit extract. Write your conclusion below the results table. [

Demonstrator/staff member will see you individually for results and table check and for any questions when you have finished your lab work/write-up!

Hand in your table at the end of the laboratory practical class with your name on it!

PRACTICAL WORKSHEET 5 **Name:**_____

A. Electrophoresis of amino acids:

	Distance from origin to "spot" centre (cm)	Movement towards anode or cathode	Charge on amino acid (+ or -)
Lysine			
Aspartic acid			
Leucine			
Unknowns			

B. TLC of carbohydrates:

Distance from origin to solvent front = _____ cm

	Distance from origin to "spot" centre (cm)	Spot colour	Rf value
Glucose			
Fructose			
2-deoxy-D-glucose			
Ribose			
Galactose			
Sucrose			
Maltose			
Unknown 1			
Unknown 2			
Unknown 3			

Unknown sugar 1: _____

Unknown sugar 2: _____ Unknown sugar 2: _____