Mucopolysaccharides by Thin Layer Chromatography

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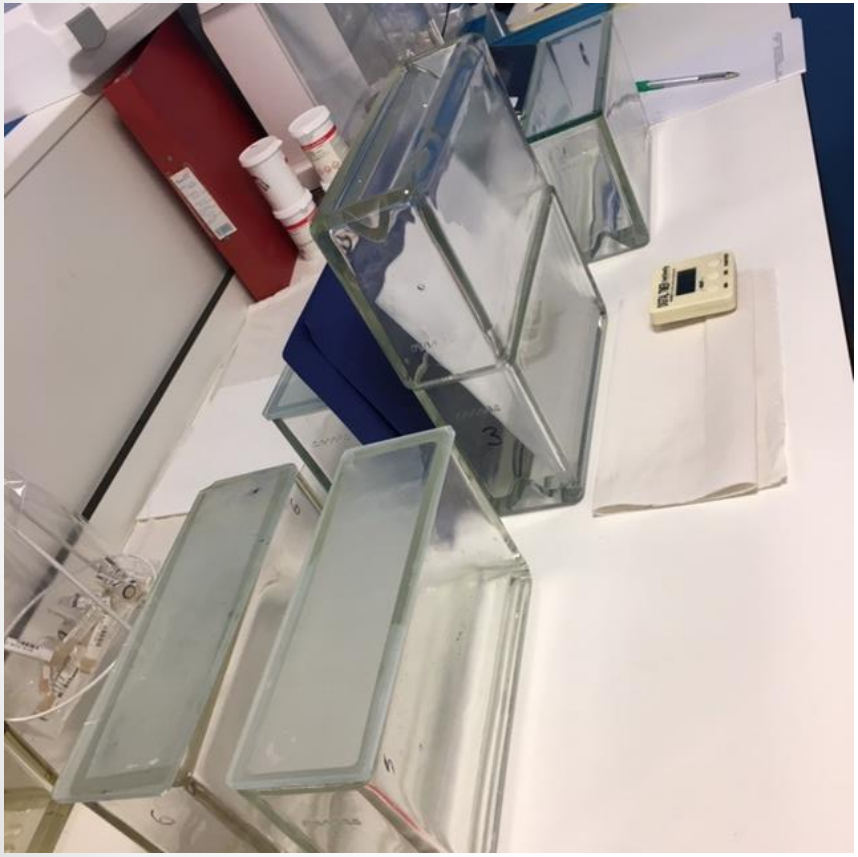
Method Overview

- Glycosaminoglycans (GAGs) extracted from 5ml urine using Alcian Blue precipitation with washes.
- Re-dissolved GAGs spotted onto a cellulose thin layer chromatography (TLC) plate.
- GAGs are separated by TLC based on the solubility of their calcium salts in the solvents.
- Plate stained with Toluidine Blue in ethanol and de-stained in acetic acid solution.

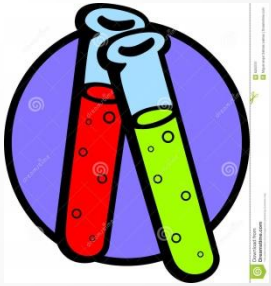
Equipment



- Chromatography tanks (for 20cm X 20cm plates).
- Macherey-Nagel micro crystalline cellulose plates.
- De-staining trays.

Reagents

- Alcian blue
- Sodium acetate buffer
- Magnesium chloride
- Sodium chloride
- Sodium Carbonate
- Toluidine Blue
- Acetic Acid
- Sodium Hydroxide
- Calcium Acetate
- Ethanol



Preparation of Standards

Prepare solutions in 0.05M NaOH at a concentration of 1mg/mL

- Chrondroitin 4-Sulphate (Chon A)
- Chrondroitin 6-Sulphate (Chon C)
- Keratan Sulphate
- Dermatan Sulphate (Chondroitin sulphate B)
- Heparan Sulphate
- Heparin Sodium: Prepare a 1 in 100 dilution of 5,000 unit/mL heparin sodium in water.

Sample Preparation

1. Add 1ml urine to 5mL of alcian blue reagent in a universal container. Mix, cap and leave **overnight** at room temperature.

2. Centrifuge and discard supernatant. Drain by inversion of the bottle, for approx 1min.

3. Add 0.2mL of 4M NaCl and 0.1mL of methanol. Mix and transfer to a conical tube and mix at intervals for 15min.

4. Add 0.1mL of Na_2CO_3 and 0.4mL of distilled water, mix and leave for 30 min at room temperature (can be left overnight)

Sample Preparation Continued

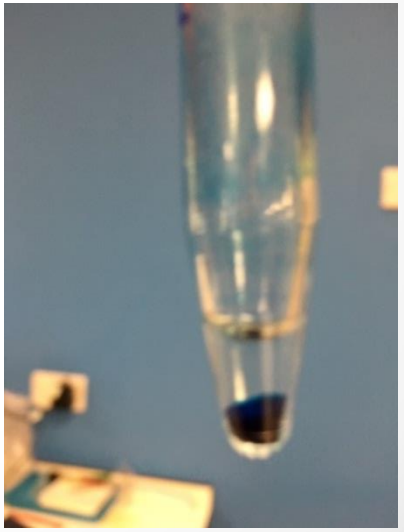
5. Centrifuge to remove the alcian blue.

6. Into a plastic conical tube add one volume of clear supernatant to three volumes of ethanol.

7. Mix well, cover and leave to stand for at least **2 days** at room temperature until a white precipitate forms.

8. Centrifuge, remove supernatant and allow precipitate to dry, usually overnight.

9. Re-dissolve in 20ul H_2O - now ready for chromatography.

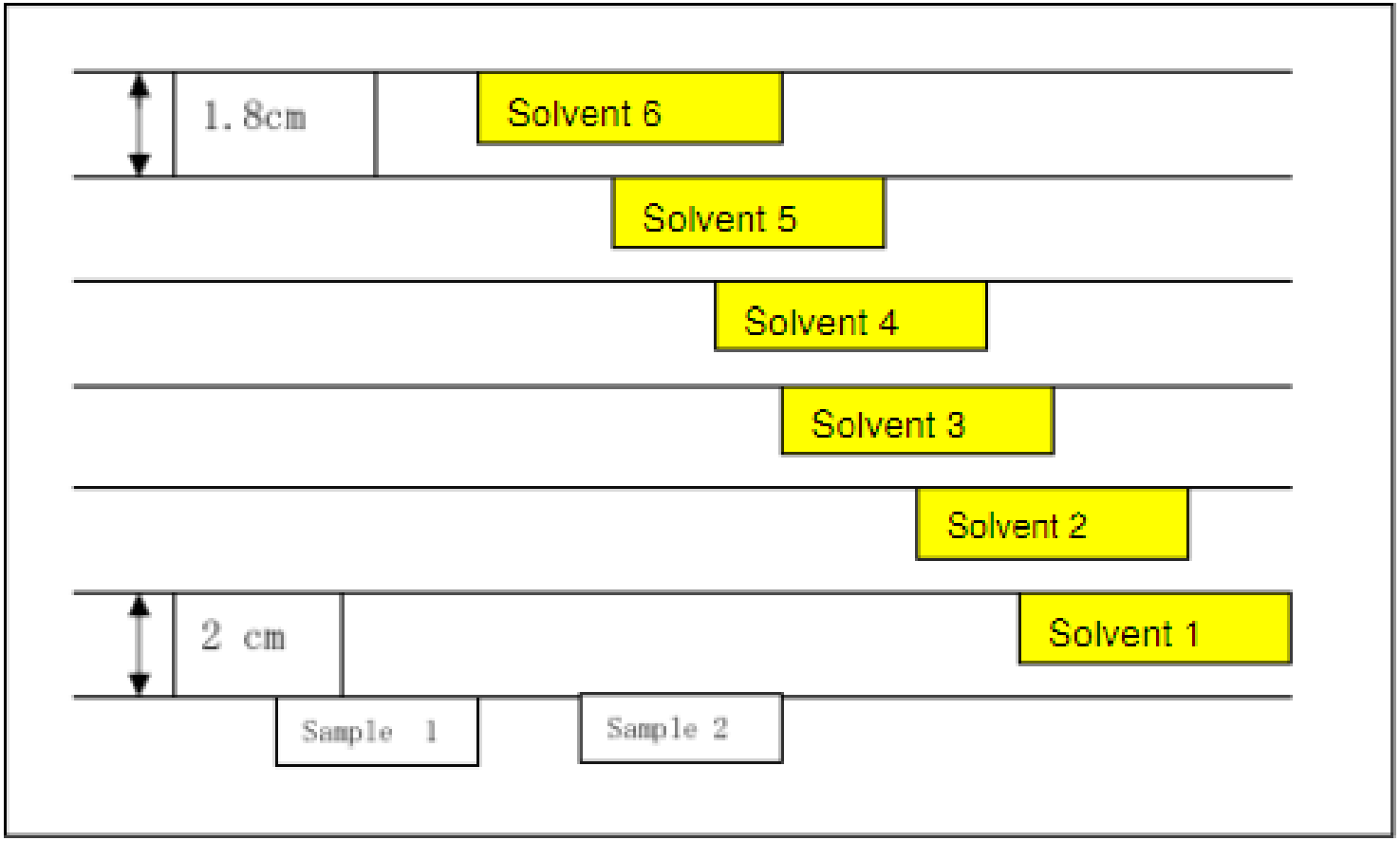


Thin Layer Chromatography

- Chromatography solvents are prepared as follows:

TANK No.	1	2	3	4	5	6
2.5% CALCIUM ACETATE (mL)	20	-	-	-	-	-
5.0% CALCIUM ACETATE (mL)	-	25	30	35	40	45
95% ETHANOL (mL)	30	25	20	15	10	5

Plate Preparation



Chromatography

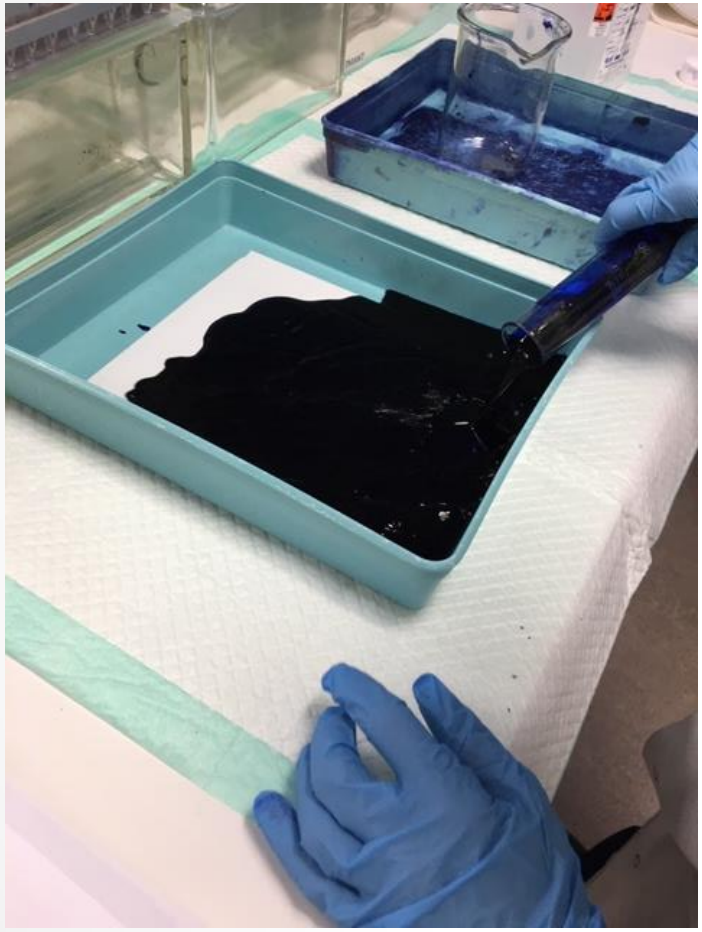
- 20µL of standard or unknown applied to sample lanes (capillary or pipette) drying with hot air between applications.
- Patient and standard spots are applied twice apart from Chondroitins A and C.
- Run the plate in each of the 6 numbered tanks, allowing each solvent to travel up to the next numbered line.
- The approximate time taken for a plate to run in each tank is given below. Keep a timer to hand!

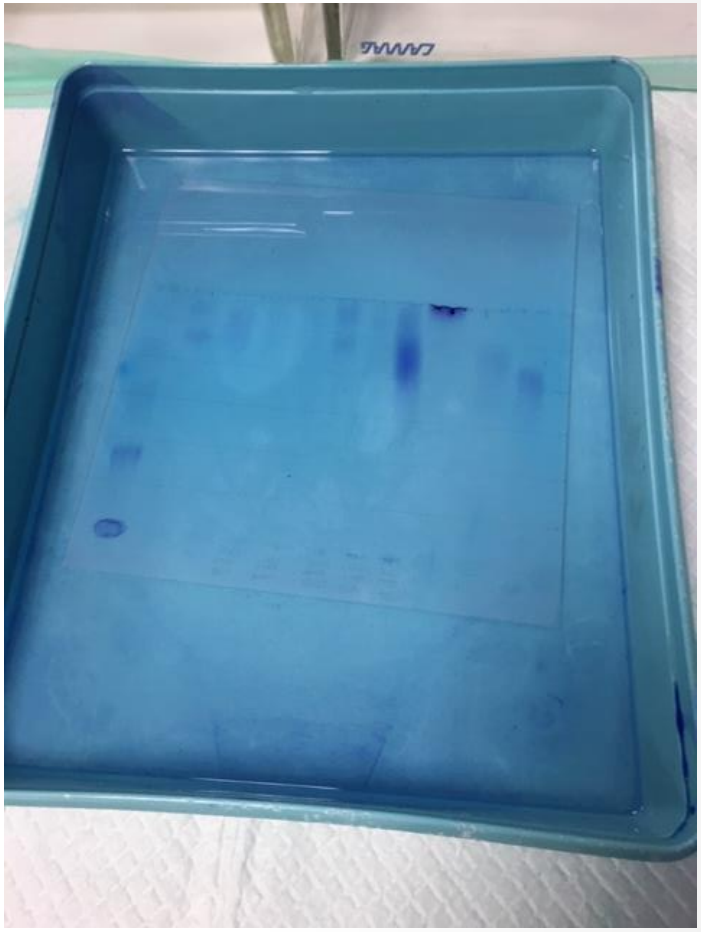
Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6
3.5 min	8.5 min	12.5 min	20.3 min	23.0 min	25.0 min

Visualisation

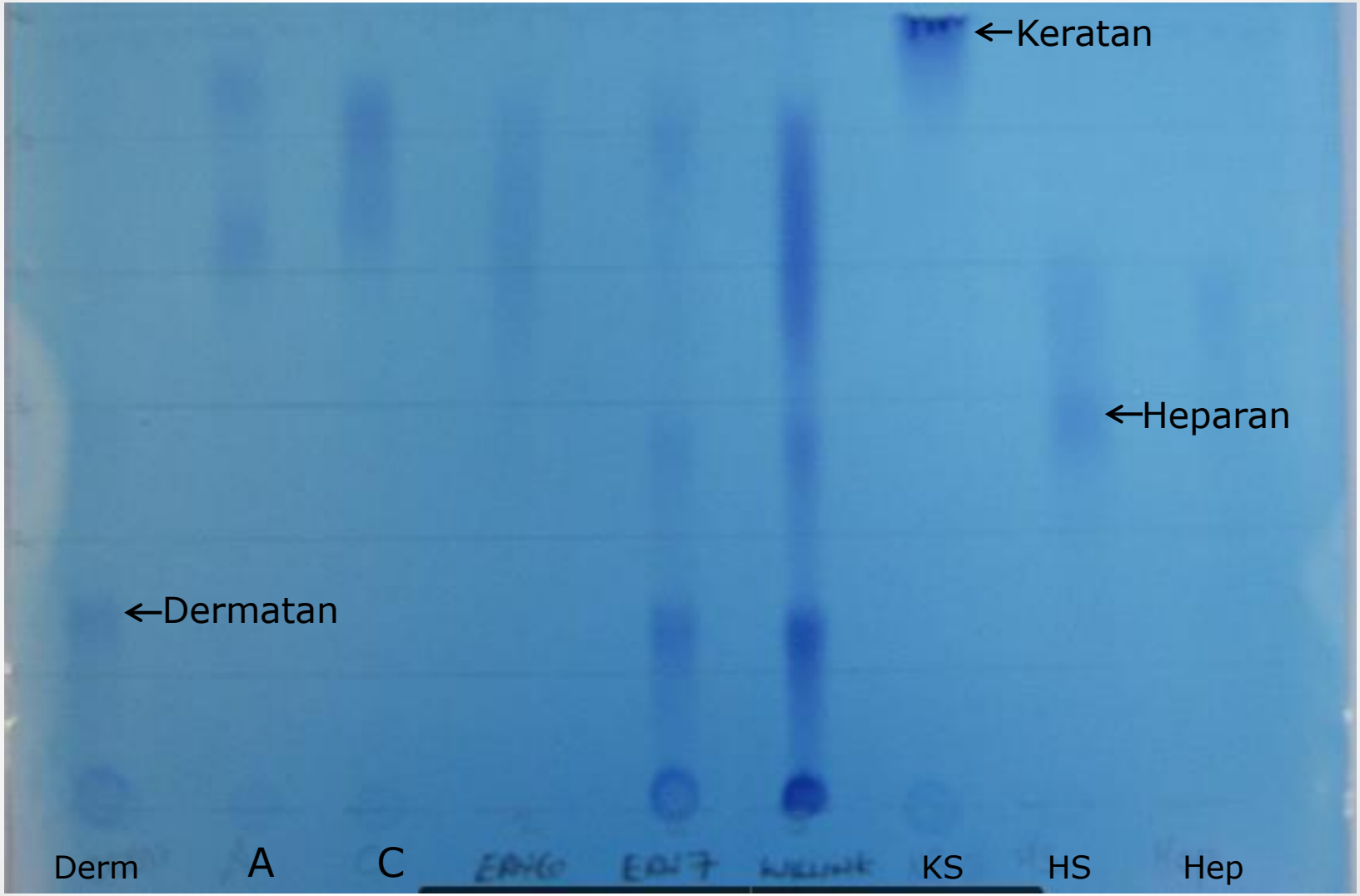
- Following chromatography the plate is dried thoroughly.
- Immediately stained in 1% ethanolic toluidine blue for 30 seconds (ensure the stain has fully dissolved)
- Then destained several times with 10% acetic acid, until the background is clear.

Visualisation

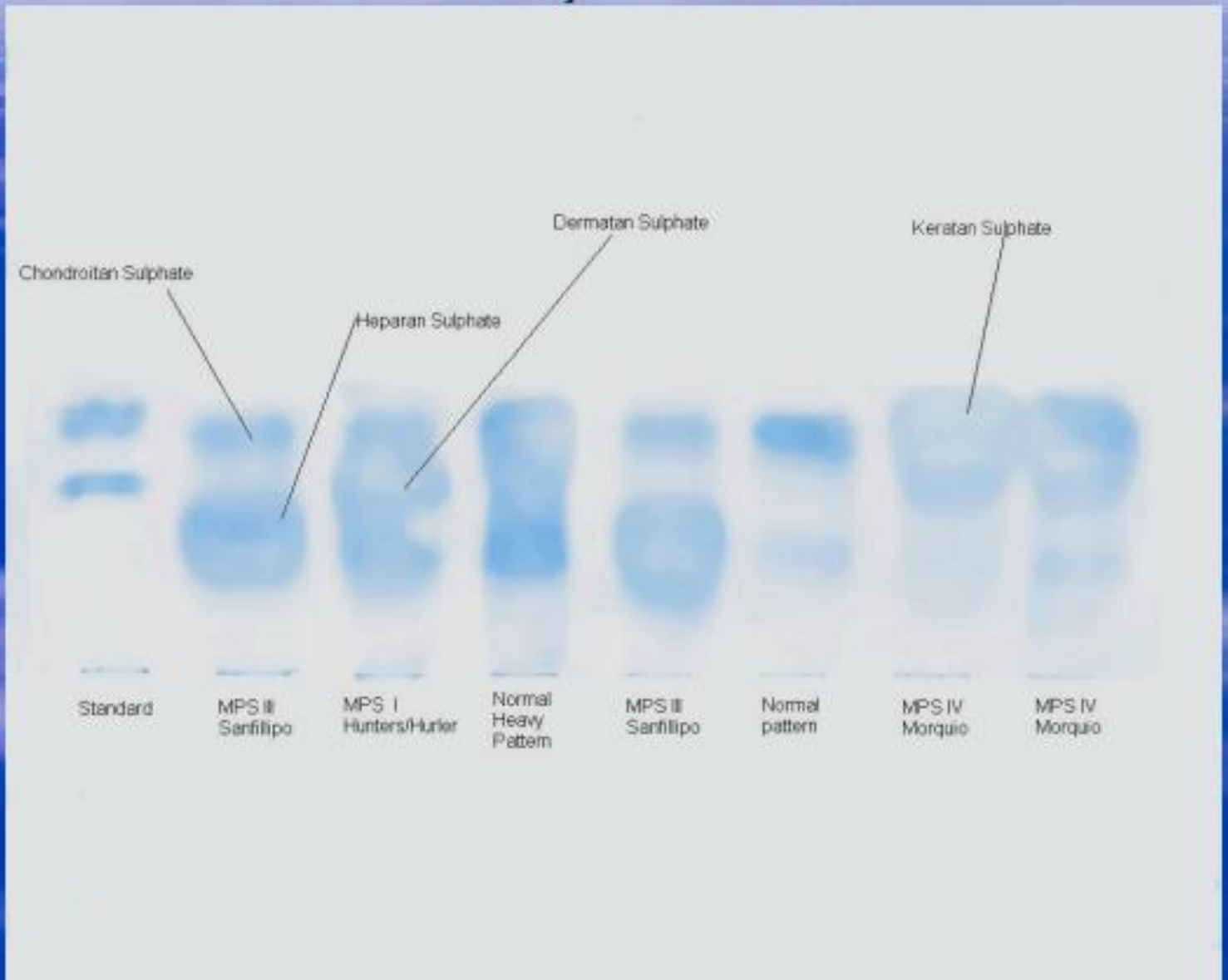




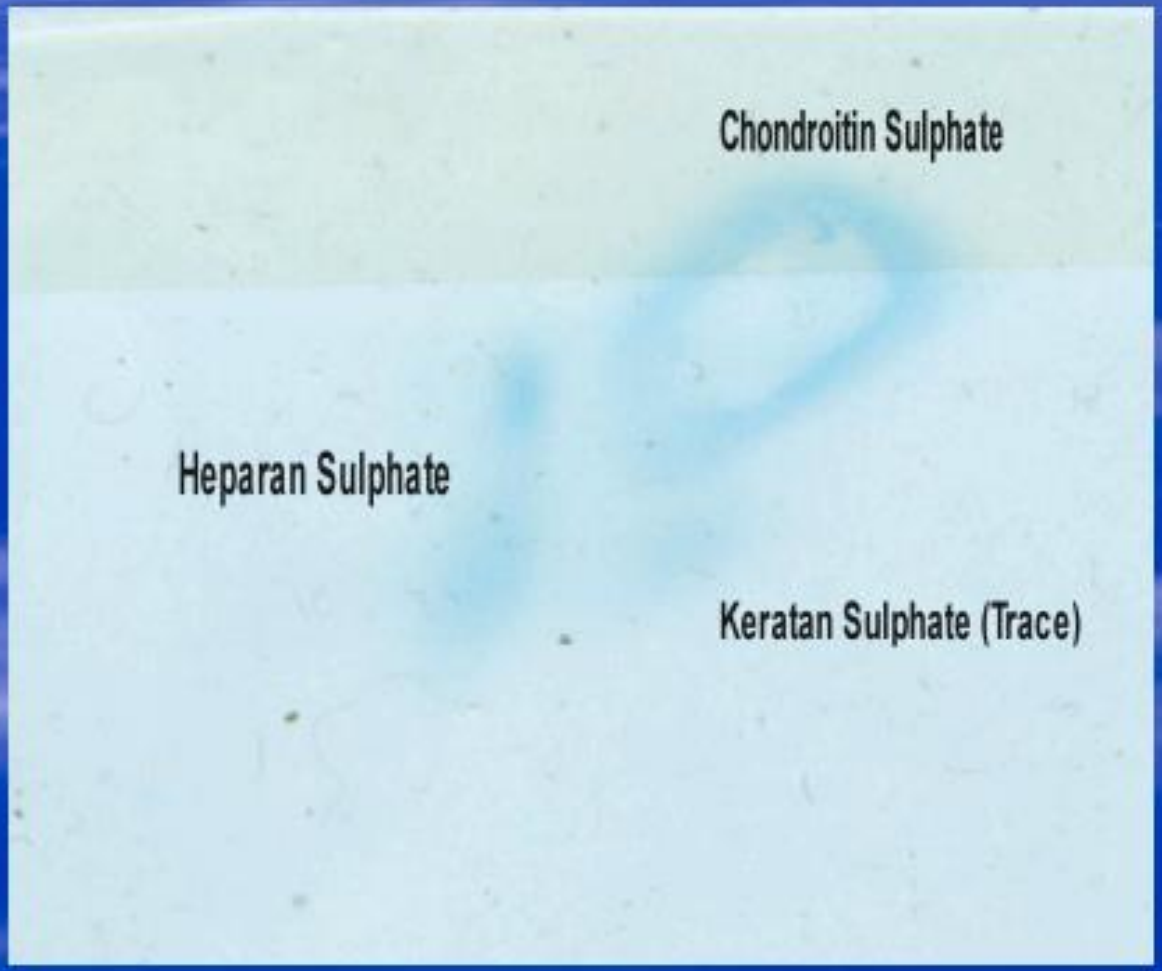
Chromatogram



One Dimensional Cellulose Acetate Electrophoresis



Two Dimensional Electrophoresis



Limitations

- **Confusing mobilities**
  - mobility of the heparan may not precisely match that of the heparan or heparin standards.
  - difficulty in distinguishing between heparan and the fastest of the spots associated with dermatan.

- **False negatives** - GAGs may be borderline elevated eg in MPSIV. Keratan may be increased but runs at solvent front where blue band "debris" from other components make it difficult to definitely exclude its presence.

- **Detection limit -** for dermatan sulphate the ability of the eye to discern a blue spot on the plate is >20mg/100mL.

- **Variable patterns in Mucopolysaccharide Diseases** - Definite diagnosis is not possible by GAG analysis alone.

- **False positives** - other connective tissue diseases may be associated with excretion of small amounts of heparan sulphate.

Practical Considerations

- Time consuming.
- Messy!
  Wear two pairs of gloves when handling dyes and protect bench.