# Paper Chromatography as Exemplified by Separation of Urocanic Acid and Deaminohistidine

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**[Abstract]** Paper chromatography is an ancient technique to separate low molecular mass compounds based on their distribution between mobile phase (solvent) and stationary phase (cellulose and cellulose-bound water). Paper chromatography has been largely replaced by thin layer chromatography and high performance liquid chromatography as the latter methods have higher resolution capability. Nevertheless due to low cost and availability of great number of protocols for separation of various compounds, paper chromatography is still a powerful analytical tool. In the current protocol this technique is exemplified by separation of urocanic acid and deaminohistidine.

## Materials and Reagents

1. Chromatography paper (Whatman No. 1)
2. Urocanic acid (MP Biomedicals)
3. Deaminohistidine (Chem-Impex International)
4. Isobutanol
5. Acetone
6. Formic acid
7. Sulfanilic acid
8. $NaNO_2$
9. HCl
10. $Na_2CO_3$
11. Pauly diazo reagent (see Recipes)

## Equipment

1. Chromatography chamber
2. Capillary tubes (Kimble Chase Kimble, catalog number: 7190010)
3. Spraying nozzle

 

**Procedure**

A. Preconditioning of chromatography chamber
   1. Prepare chromatography solvent by mixing isobutanol, acetone, formic acid, and water in 160:160:1:39 proportion (vol. by vol.).
   2. Pour the solvent into a chromatography chamber, the liquid level should be around 5 mm from the bottom. Seal the solvent-containing chamber for at least 1 h to saturate the chamber with solvent vapors.

B. Preparation of chromatography paper
   1. Scissor out a strip of chromatography paper with the appropriate dimensions. The width of the strip depends on number of samples to be analyzed (the distance between sample spots and between spots and paper edges should be at least 20 mm). The height depends on difference in $R_f$ values of separated compounds and usually is 100-400 mm.
   2. Draw a line from 20 mm from the bottom of the strip by a graphite pencil (starting line). Mark dots on this line for future sample spots.
   3. Load spots of the test samples and standards on the starting line using a capillary tube. Micro liter quantities are used to prepare spots. The diameter of the spots should be only up to a few mm. In order to get a concentrated spot, the spotting can be repeated several times. If it is necessary, wait till the spot is dried and then repeat the spotting. In the case of urocanic acid and deaminohistidine, each spot should contain about 10 nmol of one of these compounds.

C. Chromatography
   1. To develop the chromatogram, place the prepared strip into the saturated chromatography chamber and seal the chamber.
   2. Remove the strip from the chamber when solvent front has traveled up to about 20 mm from the top of the paper. Mark position of the solvent front on the strip.

D. Detection
   1. Air dry the strip. Using a spraying nozzle spray the dried strip first with the Pauly diazo reagent until the paper become humid, followed by spraying the humid strip with a 10% $Na_2CO_3$ solution until spots of imidazolyl derivatives become colored (urocanic acid – orange, deaminohistidine – red). The color is stable within several weeks. The schematic representation of the typical chromatogram can be seen in Figure 1.
   2. Calculate $R_f$ values ((distance traveled by a component)/ (distance traveled by the solvent)).

 

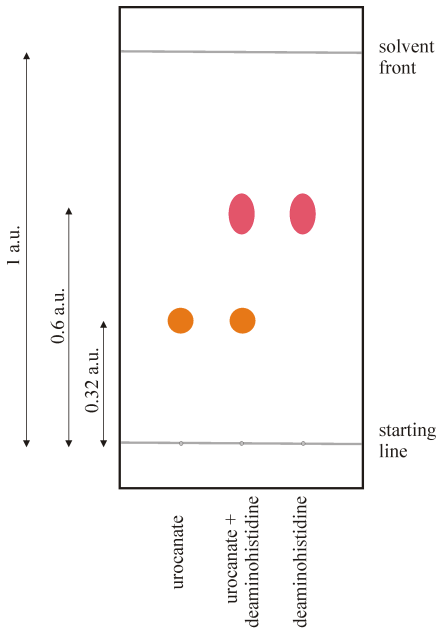


**Figure 1. Schematic representation of the separation of urocanate and deaminohistidine by paper chromatography.**

## Recipes

1. Pauly diazo reagent
   Reagent A: Dissolve 0.9 g sulfanilic acid in $H_2O$ and add 9 ml concentrated HCl (37%), and then make up to a total volume of 100 ml in $H_2O$
   Reagent B: Dissolve 5 g $NaNO_2$ in 100 ml $H_2O$
   Pauly diazo reagent: Mix 6 ml of ice cold reagent A with 6 ml of ice cold reagent B and incubate 5 min on ice. Add to the mixture additional 24 ml of reagent B and 64 ml of ice cold water. The Pauly diazo reagent can be stored for a few hours on ice.

## Acknowledgments

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## References

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