

Determination of amino acids using thin layer chromatography.

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http://tera.chem.ut.ee/~koit/arstpr/ah_en.pdf

1 Introduction

1.1 Method basics

Planar chromatography belongs to the family of chromatographic methods used for separation and determination of substances. It allows to carry out qualitative and sometimes also quantitative analysis of chemical components in complex mixtures. Substances to be determined are transferred onto the layer of sorbent as a spot or band using a capillary, microsyringe or special applicator. Sorbent may comprise of a layer (0,05...0,2 mm thick) of fine-grain material (silicagel, cellulose, aluminium oxide, ion exchange resin) on a solid support (glass, plastic, metal). Instead of sorbent, porous materials (paper, plastic etc) can be used as well.

Spots of samples are applied on the plate in a straight line called **starting line**. The mobile phase starts moving upwards due to capillary forces when the plate edge is inserted into the eluent in chromatographic chamber. While the eluent is moving the components of sample separate from each other because of the interactions between the molecules of eluent, sorbent and substances to be separated. The elution process is stopped when the eluent has traveled up the plate until 5-10 mm from the upper edge of the chromatographic plate. This eluent front is marked and this line is called **stop line**. The elution distance, h_0 , is the distance between the starting line and the stop line.

After the chromatographic separation, all the components of the analysed mixture are located somewhere between the starting line and stop line. In order to visualize the separated substances (if they are not colored) different techniques are used: chemical reactions, adding fluorescence indicators to the sorbent layer during the process of preparation of the plates or spraying the plates with fluorescent solutions and then observing under ultraviolet lamp.

As the distances travelled by the different substances differ, their mobilities can be used for qualitative analysis. Therefore, the distance between the starting line and the center of the spot of substance h_x (mm) characterizes each substance. Retention factor, R_F , provides better way to identify substances. R_F is calculated from the following formula:

$$R_F = \frac{h_x}{h_0}$$

Provided that exactly the same amount of sample is applied, the intensity of the spot can be used for quantitative determination: the bigger the amount of substance in the mixture, the more intensive is the spot. Also the size of the spot can give quantitative information – the bigger the spot, the bigger the content of this compound in the mixture. Intensity of the spots is evaluated by comparing with the intensities of analyte spots with known amounts visually or using densitometer (device for measuring optical density).

1.2 Amino acids

Proteins consist of amino acids interconnected by peptide bonds. Figure 1 depicts the general structure of α -amino acids (carboxylic acid and amino groups attached to the same carbon), which are found in living organisms.

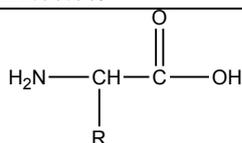


Figure 1. General structure of α -amino acids (R – variable group).

1.3 Determination of amino acids using thin layer chromatography

Mixtures of amino acids can be separated on chromatographic paper. The separated amino acids are visualized using solution of ninhydrin. Purple color develops upon reaction of amino acid with ninhydrin. Reaction in Figure 2 is one possible way ninhydrin reacts with amino acids.

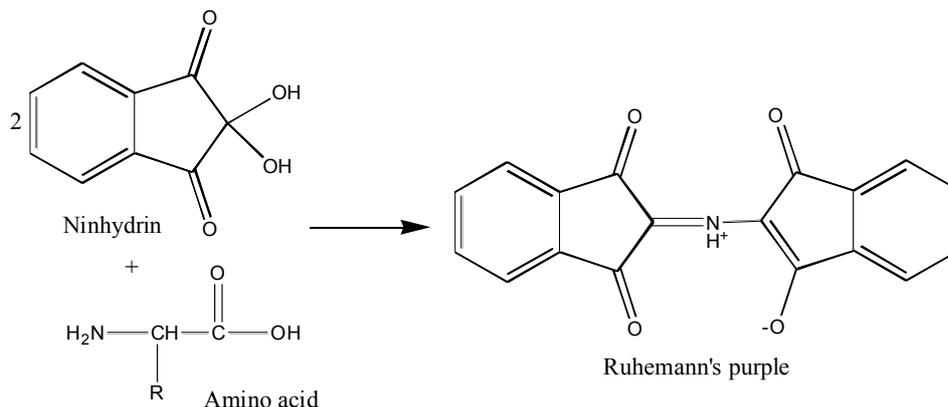


Figure 2. Development of Ruhemann's purple from ninhydrin and amino acid.

Table 1 presents structures, molar mass and R_F values for several amino acids. Note that experimentally found R_F values are strongly influenced by experimental conditions, e.g. eluent composition, humidity of the paper, temperature.

Table 1. Properties of some amino acids.

Amino acid	Structural formula	Molar mass	R_F value
Leucine	$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}-\text{CH}_3 \\ \\ \text{CH}_3 \end{array}$	131,18	0,75 – 0,90
Methionine	$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{S} \\ \\ \text{CH}_3 \end{array}$	149,21	0,45 – 0,65
Alanine	$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_3 \end{array}$	89,10	0,34 – 0,55

Amino acid	Structural formula	Molar mass	R _F value
Serine	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{OH} \end{array} $	105,10	0,19 – 0,35

2 Aim of the work

Qualitative analysis (identification) of amino acids in given mixture.

3 Instruments, chemicals and glassware

1. Eluent¹.
[Mix n-butanol, acetic acid (purity 98 – 100 %) and distilled water in volume ratio 5:1:5. Stir for 10 minutes, then let the layers separate. Use upper layer as eluent.]
2. Solution on ninhydrin.
[Dissolve 0.3 g of ninhydrin in 100 ml n-butanol. Add 3 ml of glacial acetic acid.]
3. 0.02 M solutions of amino acids (leucine, methionine, alanine and serine).
[Dissolve: 0.026 g leucine, 0.030 g methionine, 0.018 g alanine and 0.021 g serine in distilled water and bring the volume to 10 ml.]
4. Chromatographic paper with dimensions 85 x 50 mm
5. Elution chamber with approximate internal dimensions: 7 cm high and with 5.5 cm diameter. Upper edge of the chamber is grinded and the chamber is covered with two lids, the first lid has a slot for chromatographic paper.
6. Glass capillaries for spotting the samples.
7. Graduated test-tube.
8. Graphite pencil.
9. Drying oven at ~ 60° C.
10. Filter paper 10 x 10 mm or 12 cm in diameter.
11. Solution of ninhydrin in spraying bottle.
12. Ruler.
13. Scissors.
14. Rubber gloves.

4 Analytical procedure

Rubber gloves must be used during this work to avoid contamination of chromatographic paper with amino acids from skin, and for protecting skin from solvents and ninhydrin while working with the sprayer or sprayed paper. While the paper is being prepared for chromatographic analysis it should be kept on a piece of filter paper.

Mark the starting line on the paper approximately 1 cm from the edge with graphite pencil (very slight line!). Also mark the locations where the samples will be spotted. The neighboring spots should be about 8 mm apart from each other and at least 5 mm away from the paper's edge. Usually the spot of unknown substance is applied to the center of the starting line.

Before applying samples to the paper and filling the elution chamber fit the length of chromatographic paper with the height of elution chamber. Cover the chamber with the first lid and insert the chromatographic paper through its slot. With pencil mark the place where the paper extends from the slot. Remove the paper from chamber and fold it back 1 mm below the mark so that eventually the paper would hang down from the lid.

The spots of individual amino acids and sample solutions are applied to the chromatographic paper. Use separate clean and dry glass capillary for each solution. Dip the capillary into solution – some solution is drawn into the capillary. With the filled capillary touch the prepared location on chromatographic paper.

¹ Instructions in square brackets are for information only. Students don't prepare those solutions.

The spot on the paper should not be bigger than 2-3 mm. It is advised you can exercise spotting on a sheet of filter paper with distilled water.

After application of samples let the spots dry. To start the analysis, insert the chromatographic paper through the slot in the first lid and cover it with the second lid. Check if the paper reaches the eluent surface. Elution is stopped when the solvent front has traveled up the plate until 7-10 mm from the lid. Remove the paper from elution chamber and place it on a sheet of filter paper. After 2-3 minutes mark the eluent front with pencil and dry the paper in oven. When the paper is dry, take it into the fume hood and spray it with solution of ninhydrin until the paper is slightly damp. Chromatographic paper and the paper supporting it should lie at 45° angle while spraying. The chromatographic paper is again put in the drying oven for 15 min to speed up the reactions.

Use the time to wash the gloves (let the gloves be on until washed). Use distilled water to rinse the capillaries and put into oven for drying. Pour the eluent from the elution chamber into **residues bottle** and let the chamber dry.

Remove chromatographic paper from drying in the oven, draw the contours and centers of the chromatographic bands. Calculate R_F values by the method described above. Compare retention of standard substances and components in sample and determine which amino acids were present in the sample.

5 Results

Names of amino acids found in the mixture.