Lab (2): Quantitative amino acids estimation by ninhydrin method

Aim:

- Determination of amino acids quantity using ninhydrin reaction.

Introduction:

Amino acid analysis allows for amino acid quantitation of free amino acids, as well as amino acids released from macromolecules such as peptides, proteins or glycoproteins.(1) The rapid and accurate quantification of amino acid is critical to understanding the underlying biochemistry of multiple physiological and disease state, food science, drug samples and others. (1,2)

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample, i.e. HPLC. (3)

The ninhydrin reaction, one of the most important method of detecting amino acids, both technically and historically, has been conventionally used to detect their microgram amounts. When amino acids with a free alpha amino group are treated with an excess of ninhydrin, they yield a purple colored product. (4) Although this is a fast and sensitive test for the presence of alpha-amino acids, because of the non-selectivity, it cannot be used to analyze the relative individual contents of a mixture of different amino acids.(5) Several other convenient reagents are available which can react with the alpha amino group to form colored or fluorescent derivatives. These include fluorescamine, dansyl chloride, dabsyl chloride, etc., used in the detection of trace amounts of amino acids at the nanogram level. (4)

The primary amino groups react with ninhydrin to form the purple colour dye now called Ruhemann's purple (RP) was discovered by Siegfried Ruhemann in 1910. Under appropriate conditions, the color intensity produced is proportional to the amino acid concentration. In the quantitative estimation of amino acid using Ninhydrin reagent, the absorbance of the Ruhemann's purple formed by the reaction at 570 nm is measured, whereas for imino acids, the absorption happens at 440 nm. (6)

Since there is a proportional relationship between the concentration and absorbance, a standard curve could be constructed to determine an unknown concentration of an amino acid sample. The standard curve (also called calibration curve) is a type of graph used as a quantitative research technique that shows the relationship between different known concentrations of a substance and the absorbance at a specific wave length. Standard curve is most commonly used to determine the concentration of a substance (unknown), using serial dilution of solutions (standard solutions) of known concentrations.(7)

- PAUSE AND THINK ➔ How an unknown concentration could be determined by knowing its absorbance at given wavelength?

The determination of unknown concentration from the standard curve is done by drawing a line parallel to the X- axis from the point on the Y axis that corresponds to the absorbance of the unknown. This line will be made to intersect the standard curve drawn and is extended vertically such that it meets the X-axis and the concentration of unknown is read from the X-axis. A typical standard curve is depicted in the Figure 1. (6)

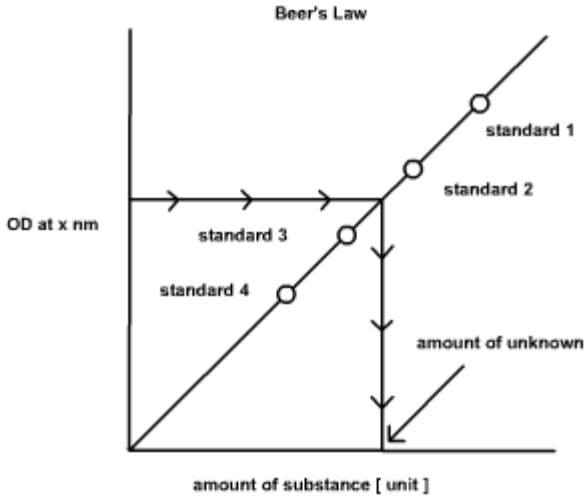

Figure 1. A standard curve showing the relation between the absorbance of different concentrations of a substance.(6)

Principle:

At nuetral pH, ninhydrin destroys each primary α-amino acid and also reacts with the released NH_3 to form a deep purple chromogen referred to as Ruhemann's purple, which has a maximum absorption at about 570 nm. The reaction with proline and other imino acids yields a yellow- orange product at neutral pH, as the cyclised N-group is not released. The intensity of the color resulted is linearly proportional to the concentration of the amino acids present in the solution.(8)

Materials:

Chemical

Standard amino acid stock solution (500 µg/ml), 8% w/v of ninhydrin reagent, 50% v/v ethanol, distal water.

Equipment and Glassware

Test tubes, rack, pipette, pipette pump, aluminum foil, plastic cuvette, spectrophotometer, water bath.

Protocol:

1. Set up 7 test tubes as following:

Tube	Standard amino acid solution (500 µg/ml) (ml)	Distilled water (ml)	Unknown sample	Ninhydrin reagent (ml)
Blank	-	4		1
A	1.2	2.8		
B	1.6	2.4		
C	2	2		
D	2.4	1.6		
E	2.8	1.2		
Unknown sample	-	-	4	

2. Mix the contents of the tubes by vertexing/shaking the tubes.
3. Cover the mouth of the tubes with aluminium foil.
4. Place all the test tubes in boiling water bath for 15 minutes.
5. Cool the test tubes in cold water, the add 1 ml of ethanol to each test tube and mix well.
6. Record the absorbance of all tubes against the blank at 570 nm using a colorimeter (spectrophotometer).

PAUSE AND THINK ➔ What the blank should contain? Why?

7. Calculate the amino acid concentration for each standard amino acid solution using $C_1 \times V_1 = C_2 \times V_2$ formula.
8. Plot standard curve for absorbance against amino acids concentration (µg/ml) using results for solutions (A-E).
9. From the standard curve, estimate the concentration of the amino acids present in your unknown sample.

Results:

Test tube	Amino acid concentration [µg/ml]	Absorbance at 570 nm
Blank		
A		
B		
C		
D		
E		
Unknown sample	_____	

Supporting materials:

- A video explains the mechanism of reaction between ninhydrin and amino acid: https://www.youtube.com/watch?v=P-iK4QUU9t0

- A video shows the practical steps of the experiment step by step:
  -Part 1: https://www.youtube.com/watch?v=wvVYF8T7uiE
  -Part 2: https://www.youtube.com/watch?v=B4EgYeFqV5Q

References:

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2. http://www.waters.com/waters/library.htm?lid=134820198&locale=en_SA
3. http://vlab.amrita.edu/?sub=3&brch=63&sim=156&cnt=1
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