14.1 Determination of allantoin - Colorimetric method

Chemistry

The manual method adapted to measure allantoin is based on the calorimetric method described by Young and Conway (1942). In this procedure, allantoin is hydrolyzed firstly under a weak alkaline condition at 100ºC, to allantoic acid which is further degraded to urea and glyoxylic acid in weak acid solution. The glyoxylic acid then reacts with phenyl hydrazine hydrochloride to produce a phenyl hydrazone of the acid. The product can then form an unstable chromophore with potassium ferricyanide. The color is read at 522 nm.

Apparatus

- Spectrophotometer
- Boiling water bath. If you have a temperature controlled water bath, you may use polyethylene glycol (PEG MW 400) solution instead of water in the bath and set the temperature at 100ºC. The temperature can be better controlled since PEG has a boiling point greater than 100ºC.
- Ultrasonic bath (Fig. 14.1)

Fig. 14.1 Ultrasonic bath

Chemicals

- Sodium hydroxide (NaOH) 0.5 M
- NaOH 0.01 M
- Hydrochloric acid (HCl) 0.5 M
- Phenyl hydrazine hydrochloride 0.023 M freshly prepared before use.
- Potassium ferricyanide 0.05 M freshly prepared before use.
- Concentrated hydrochloric acid (11.4 N) cooled at -20ºC at least 20 min before use.
- Alcohol bath, 40% (v/v) alcohol, kept at -20ºC. You may use 40% NaCl solution instead of alcohol solution.
- Allantoin

Standards

Prepare a stock allantoin solution 100 mg/L. Dilute it to give working concentrations of 10, 20, 30, 40, 50 and 60 mg/L.

1) Weigh 50 mg of allantoin and transfer it to a 500 ml volumetric flask. Dissolve in about 100 ml 0.01 M NaOH, and top up to volume with distilled water. The addition of NaOH is only to help to dissolve allantoin.
2) To prepare 50 ml of the working standards 10, 20, 30, 40, 50 and 60 mg/L respectively pipette 5, 10, 15, 20, 25 and 30 ml of stock solution into 50 ml volumetric flasks and make up to volume with distilled water. If you do this analysis routinely, it is better to prepare the standard solution in larger volumes.
3) Store each working standards as small aliquots in the freezer. Only the necessary quantities are thawed and any leftover discarded. This ensures that fresh standards are used for each analysis run.

Preparation

Before the analysis:

1) Put the alcohol bath into the freezer over night.
2) Put the concentrated hydrochloric acid into the freezer just before the beginning of the analysis.
3) Switch on the water bath.
4) Check the samples. If any precipitate is visible, place the samples in an ultrasonic bath for 20 min. to break up the particles.
5) Prepare the fresh solutions of Phenyl hydrazine hydrochloride and Potassium ferricyanide.

Phenyl hydrazine hydrochloride and potassium ferricyanide solution:

Prepare solutions of phenyl hydrazine hydrochloride and potassium ferricyanide on the day of analysis (keep these solutions in fridge before use). Fifty ml is enough for 10 samples in duplicate. Weigh 0.1663 g of phenyl hydrazine hydrochloride, dissolve in a small beaker and transfer to a 50 ml volumetric flask. Top up to volume with distilled water. Weigh 0.835 g potassium ferricyanide and transfer to a 50 ml volumetric flask, dissolve and make up to volume with distilled water.

Procedure

This procedure requires critical timing of the reactions. The reading of standards and samples OD must be done within a shortest possible time-span, since OD decrease with time. Therefore, no more than 10 samples in duplicate should be processed in each run. A set of standards and a blank (using distilled water) in duplicate are processed.

- Pipette 1 ml of sample, standard or distilled water (blank) into 15 ml tubes.
- Add 5 ml of distilled water.
- Add 1 ml of 0.5 M NaOH.
- Mix the contents of the tubes by vortexing.
- Put the tubes in the boiling water bath for 7 min.
- Remove from the boiling water and cool the tubes in cold water.
- Add to each tube 1 ml of HCl (0.5 M). The pH after adding the HCl must be in the range 2-3.
- Add 1 ml of the phenyl hydrazine solution. Mix and transfer the tubes again to the boiling water for exactly 7 min.
- Remove from the boiling water and dump it immediately into the icy alcohol bath for several min.
- Pipette 3 ml of concentrated HCl (operate in a fume cupboard) and 1 ml of Potassium ferricyanide. Perform this for all samples within a shortest possible span. Start the timer.
- Mix thoroughly and transfer some to 4.5 ml cuvettes at room temperature.
- Read the absorbance at 522 nm after exactly 20 min. on the timer. Once started, do it as quickly as possible (because the color will disappear). It is important that

OD for samples and standards be read at a shortest possible time span.

Calculation

Standard curve:

The standard curve should be linear. Therefore, we can fit a linear regression between the known allantoin concentrations (standards) (X) and the corresponding OD (Y). The slope of the line is usually 0.16-0.18. Calculate the concentration of the unknown based on this equation.

Reference: Young, E.G and Conway, C.F., 1942. On the estimation of allantoin by the Rimini- Schryver reaction. J. Biol. Chem., 114: 243-248.