

**Enzymatic Assay of HESPERIDINASE
(EC 3.2.1.40)**

PRINCIPLE:

Hesperidin + H₂O $\xrightarrow{\text{Hesperidinase}}$ Reducing Sugar (measured as glucose)

CONDITIONS: T = 40°C, pH = 3.8, A_{540nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 200 mM Sodium Phosphate Solution
(Prepare 100 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751.)
- B. 100 mM Citric Acid Solution
(Prepare 100 ml in deionized water using Citric Acid, Free Acid, Monohydrate, Sigma Prod. No. C-7129.)
- C. McIlvaine Buffer, pH 3.8 at 40°C
(Prepare 100 ml using Reagent A. Adjust to pH 3.8 at 40°C with Reagent B.)
- D. 666 mM Sodium Hydroxide Solution (NaOH)
(Prepare 100 ml in deionized water using Sodium Hydroxide, Anhydrous, Sigma Prod. No. S-5881.)
- E. 2.0 mM Hesperidin Substrate Solution (Hesperidin)¹
(Prepare by dissolving 120 mg of Hesperidin, Sigma Prod. No. H-5254, in 30 ml of Reagent D. Add 40 ml of Reagent C and adjust to pH 3.8 with 1 M HCl.)
- F. Hesperidinase Enzyme Solution
(Immediately before use, prepare a solution containing 0.03 - 0.06 unit/ml of Hesperidinase in cold deionized water.)

**Enzymatic Assay of HESPERIDINASE
(EC 3.2.1.40)**

REAGENTS: (continued)

- G. 16 mM Copper Sulfate, 1.26 M Sodium Sulfate, 226 mM Sodium Carbonate, 190 mM Sodium Bicarbonate, and 43 mM Sodium Potassium Tartrate Solution (Copper Soln) (Prepare 1 liter in deionized water using Cupric Sulfate Pentahydrate, Sigma Prod. No. C-7631, Sodium Bicarbonate, Sigma Prod. No. S-8875, Sodium Sulfate, Anhydrous, Sigma Prod. No. S-9627, Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127, and Sodium Potassium Tartrate Tetrahydrate, Sigma Prod. No. S-2377.)²
- H. 40 mM Molybdic Acid, 19 mM Arsenic Acid and 756 mM Sulfuric Acid Solution (Ars-Mol) (Prepare 1 liter in deionized water using Molybdic Acid, Ammonium Salt Tetrahydrate, Sigma Prod. No. M-0878, Arsenic Acid, Sodium Salt, Sigma Prod. No. A-6756, and Sulfuric Acid, Sigma Prod. No. S-1526.)³
- I. 5.55 mM Glucose Standard Solution (Std) (Use Glucose Standard Solution, Sigma Stock No. 635-100.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable test tubes:

	<u>Test</u>	<u>Blank</u>
Reagent E (Hesperidin)	4.00	4.00

Equilibrate to 40°C. Then add:

Deionized Water	-----	1.00
Reagent F (Enzyme)	1.00	-----

Mix by swirling and incubate at 40°C for exactly 30 minutes.⁴ Then add:⁵

	<u>Test</u>	<u>Test Blank</u>	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	<u>Std Blank</u>
Test Solution	1.00	----	----	----	----	----	----
Test Blank Soln	----	1.00	----	----	----	----	----
Deionized Water	----	----	0.95	0.90	0.85	0.80	1.00

Reagent I (Std)	----	----	0.05	0.10	0.15	0.20	----
Reagent G (Copper Soln)	1.00	1.00	1.00	1.00	1.00	1.00	1.00

**Enzymatic Assay of HESPERIDINASE
(EC 3.2.1.40)**

PROCEDURE: (Continued)

Immediately mix by swirling. Place a marble over the top of the tubes and transfer the tubes to a boiling water bath. Incubate for 10 minutes. Remove from the boiling water bath and allow to cool to room temperature. Then add:

Reagent H (Ars-Mol)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
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Shake or vortex the tubes until the foaming stops and any precipitate present is dissolved. Then add:

Deionized Water	10.00	10.00	10.00	10.00	10.00	10.00	10.00
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Mix by swirling and transfer the solutions to suitable⁶ cuvettes. Record the A_{540nm} for Test, Blank and Standards, using a suitable spectrophotometer.

CALCULATIONS:

Standard Curve:

$$\Delta A_{540nm} \text{ Std} = A_{540nm} \text{ Std} - A_{540nm} \text{ Std Blank}$$

Prepare a standard curve by plotting the ΔA_{540nm} of the Standard vs the μmoles of glucose.

Sample Determination:

$$\Delta A_{540nm} \text{ Sample} = A_{540nm} \text{ Test} - A_{540nm} \text{ Test Blank}$$

Determine the μmoles of glucose liberated using the Standard Curve.

$$\text{Units/ml enzyme} = \frac{[\mu\text{moles of reducing sugar (as glucose) liberated}](df)(5)}{(30)(1)(1)}$$

- df = Dilution factor
- 5 = Volume (in milliliters) of reaction mix
- 30 = Time (in minutes) of assay per the Unit Definition
- 1 = Volume (in milliliter) of enzyme used
- 1 = Volume (in milliliter) of reaction mixture used in the colorimetric determination of the reducing sugar

**Enzymatic Assay of HESPERIDINASE
(EC 3.2.1.40)**

CALCULATIONS: (continued)

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will liberate 1.0 μ mole of reducing sugar (as glucose) from hesperidin per minute at pH 3.8 at 40°C.

FINAL ASSAY CONCENTRATIONS:

In a 5.00 ml reaction mix, the final concentrations are 1.6 mM hesperidin and 0.03 - 0.06 unit hesperidinase. (Note: Concentration of the buffers are not exact due to the method of preparation of the McIlvaine's Buffer.)

REFERENCE:

Sanchez, M.A., Romero, C., Manjon, A., and Iborra, J.L. (1987) *Biotechnology Letters* **9**, 871-874

NOTES:

1. Hesperidin substrate solution only remains soluble at pH 3.8 (Assay Conditions) for 5 - 20 minutes. Immediately upon titrating the pH of the hesperidin solution to pH 3.8, this solution should be dispensed into reaction vessels (4.0 ml each).
2. Sodium Sulfate, Sodium Carbonate, and Sodium Potassium Tartrate are dissolved in approximately 500 ml of deionized water. Cupric Sulfate is dissolved in approximately 100 ml of deionized water and is slowly added to the above solution to avoid precipitation. Sodium Bicarbonate is dissolved first in deionized water and then added to the above solution. Dilute the solution to 1 liter. If a precipitate forms, it should be removed by filtration prior to use. Store in an amber bottle at room temperature and avoid exposure to direct sunlight.

**Enzymatic Assay of HESPERIDINASE
(EC 3.2.1.40)**

NOTES: (continued)

3. Molybdcic Acid is dissolved in approximately 300 ml of deionized water. Add Sulfuric Acid slowly. Caution, this is an exothermic reaction! Arsenic Acid is dissolved in approximately 300 ml of deionized water and is added to the above solution. The solution is diluted to a total volume of 1 liter and incubated at 37°C for 48 - 72 hours. Stir vigorously for 1 hour prior to each use. Store in an amber bottle at room temperature and avoid exposure to direct sunlight. The solution expires six months after preparation.
4. Since the hesperidin substrate will precipitate out of solution, the reaction vials should be swirled often during the reaction time to keep the substrate in suspension.
5. Copper Solution (Reagent G) should be dispensed into vials ahead of time to facilitate stopping the reaction.
6. This solution should be centrifuged prior to reading in spectrophotometer.
7. This assay is based on the cited reference.
8. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.