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MILK CLOT UNIT ANALYTICAL METHOD (MCU)

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A. Principle: This assay is based on the proteolytic hydrolysis of a buffered milk substrate at 40°C. Enzymatic activity is related to the time required to clot 25 ml of substrate. The enzymatic activity is always compared to an enzyme standard. This standard eliminates the batch to batch and day to day variability with the milk substrate.

B. Equipment:

1. Constant Temperature water bath at 40°C ± 0.1°C
2. pH Meter
3. Analytical Balance
4. Volumetric Flasks
5. Volumetric Pipettes
6. Automatic Pipetter
7. Stopwatch: 30 or 60 minutes ± 0.01 seconds
8. Magnetic Stirrer.
9. 50 ml screw cap tubes

C. Safety Precaution:

1. Utilize standard laboratory safety practices.
2. Acetic Acid: Pipetting should be performed in the fume hood.

D. Reagent and Reagent Preparation: Volumes may be adjusted depending on requirements.

1. <u>Concentrated Buffer</u>: (Stock Reagent)
   a. <u>Acetic Acid Solution</u>: Add 61.0 grams of glacial acetic acid to approximately 800 ml. of distilled water in a 1 L volumetric flask. Dilute to volume with distilled water.
   b. <u>Sodium hydroxide (1N NaOH):</u> Dilute 100 ml of Stock (purchased) 10N NaOH to 1000 ml with distilled water in a volumetric flask or dissolve 40.0 g of Sodium Hydroxide (anhydrous) in distilled water, quantitatively transfer the solution to a 1000 ml volumetric flask and dilute to volume.
   c. Using a standardized pH meter, add 1N NaOH to 500 ml of acetic acid solution, with constant stirring, to give a final pH of 4.50.
2. <u>Dilute Buffer solution</u>: Mix 114 ml of concentrated buffer with distilled water to make a total volume of 850 ml. <u>Prepare fresh daily when making milk substrate.</u>
3. <u>Milk Substrate</u>: Stable for one week when stored at 4-8° C.
   a. Slowly mix 200 grams of instant nonfat milk into 850 ml of dilute buffer solution in a 2000 ml beaker using a magnetic stirring.
   b. Mix for 30 minutes making sure that all the powder is mixed, and no lumps remain.

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c. Add five drops of toluene and stir for 5 additional minutes.
d. Allow milk to sit covered for at least one hour to make sure any foaming has dissipated.
e. Filter through glass wool that has been loosely packed into a funnel.
f. Allow this solution to stand for four hours at refrigeration before using.
g. The substrate is stable for one week when stored under refrigeration.

4. Enzyme Buffer: (2L)
   a. Place a 2000-ml or greater beaker with a stir bar on the magnetic mixer.
   b. Add approximately 1600-1700 ml of distilled water to the beaker.
   c. Quantitatively add:
      14.20 g Anhydrous Sodium Phosphate
      12.20 g L-Cysteine
      28.00g EDTA
   d. Allow all ingredients to stir until dissolved.
   e. pH the Buffer solution (original pH should be about 5.7) to 6.0 with the 1 N NaOH (approximately 20 ml will be needed).
   f. Quantitatively transfer the buffer to a 2000 ml volumetric flask and dilute to volume with distilled water.
   g. Repeat if necessary.

E. Procedure:

1. Milk Substrate Standardization: Using Enzyme Development's powder standard, accurately weigh the enzyme on an analytical balance to yield a final enzyme dilution that gives a clotting end point of 2.0 to 3.5 minutes.
   a. The standard is accurately weighed (assigned by lot#) on an analytical balance and is diluted with the Enzyme buffer.
   b. The beaker is placed on a stir plate, and the solution is stirred for at least 5 minutes.
   c. This solution is then quantitatively transferred to a 100 ml volumetric flask.
   d. Use the average of the three clotting times (**ST**) to determine (**M**), the milk clotting factor.

2. Enzyme Preparation:
   a. Prepare an enzyme solution in enzyme buffer so that 2.0 ml of the final dilution will give a clotting time equal to the standard clotting time (between 2.5 to 3.5 min.)
   b. Calculating enzyme preparation:

$$\text{Enzyme weight (g)} = \frac{M}{ST \times \text{Target activity}} \div 2^{*}$$

* 2 ml of enzyme preparation injected

3. Enzyme Evaluation:
   a. Pipette 25.0 ml of substrate into a series of test tubes (50 ml screw cap tube).
   b. Allow at least two tubes for each enzyme sample.
   c. Stopper each tube and equilibrate for 20 minutes in a 40°C ± 0.1°C water bath.

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d. At zero time, pipette 2.0 ml of enzyme solution into a test tube, while simultaneously starting stopwatch, and mix completely by inverting the stoppered tube slowly four times.
e. Return the tube to the water bath and rotate the tube slowly in an almost horizontal position and observe the milk film that drains from the tube walls.
f. The milk film will begin to thicken approximately 20 seconds before the end point.
g. The end point is the exact moment that "pebbling" begins. (There will be very small beads of coagulated milk along the glass – must look very carefully to observe when this "clotting" begins.)
h. Stop the stopwatch and record the time in minutes (record to two places after the decimal point).

F. Calculations:

1. Milk Factor: Must be determined DAILY for each batch of Milk Substrate and can be used for 8 hours.
   a. Example: 0.700 g of EDC Standard, assaying 135 MCU/mg (Actual activity and g weight assigned by lot), was diluted to 100 ml with enzyme buffer. 2.0 ml of this dilution was added to 25.0 ml of equilibrated substrate. The average clotting time for three separate determinations was 3.05 minutes.

Example: = M = 135 x 0.014 x 3.05
M = 5.765

Milk Factor = M = (know standard's activity)(enzyme concentration)(standard time)

Where:
135 = MCU/mg activity of EDC Standard (Actual activity assigned by lot)
0.014 = grams of enzyme added to the substrate in 2.0 ml aliquot (g wt. x 2)
3.05 = (ST) Average standard clotting time in minutes

2. Sample Activity:

$$\text{MCU/mg} = \frac{M}{T \text{ (minutes)} \times W(g)}$$

Where:
M = Milk Factor
T = Clotting time of sample
W = Grams of enzyme added to the substrate in 2.0 ml aliquot (g wt. x 2)

G. Testing Parameters:

1. Enzyme sample clotting time must be ±10/100 minutes of the average standard time.

H. Reference:

1. A. K. Balls and S. R. Hoover, *J. Biol. Chem.* 121, 737 (1937)

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