ANNEX II

METHODS OF ANALYSIS RELATING TO THE COMPOSITION OF CERTAIN PARTLY OR WHOLLY DEHYDRATED PRESERVED MILK PRODUCTS INTENDED FOR HUMAN CONSUMPTION

METHOD 8: DETERMINATION OF PHOSPHATASE ACTIVITY (ASCHAFFENBURG AND MÜLLEN PROCEDURE)

1. SCOPE AND FIELD OF APPLICATION

This method describes the determination of phosphatase activity in:

— dried high fat milk or high fat milk powder,
— dried whole milk or whole milk powder,
— dried partly skimmed milk or partly skimmed-milk powder,
— dried skimmed milk or skimmed-milk powder.

2. DEFINITION

The phosphatase activity of dried milks is a measure of the quantity of active alkaline phosphatase present in the product. It is expressed as the quantity of p-nitrophenol in micrograms liberated by 1 ml of the reconstituted sample, under the conditions described.

3. PRINCIPLE

The reconstituted sample is diluted with a buffer substrate at pH 10,2 and incubated at a temperature of 37 ^{o}C for two hours. Any alkaline phosphatase present in the sample will, under these circumstances, liberate p-nitrophenol from added disodium p-nitrophenyl phosphate. The p-nitrophenol liberated is determined by direct comparison with standard colour glasses in a simple comparator using reflected light.

4. REAGENTS

4.1. Sodium carbonate-bicarbonate buffer solution.

Dissolve 3,5 g of anhydrous sodium carbonate and 1,5 g of sodium bicarbonate in water and dilute to 1 000 ml in a volumetric flask with water.

4.2. Buffer substate.

Dissolve 1,5 g of disodium p-nitrophenylphosphate in sodium carbonate-bicarbonate buffer (4.1) and dilute to 1 000 ml in a volumetric flask with buffer (4.1).

This solution is stable if stored in a refrigerator ($\leq$ 4 ^{o}C) for one month but a colour control test should be carried out on such stored solutions — see 6, precaution number 3.

4.3. Clarification solutions.

4.3.1. Zinc sulphate solution.

Dissolve 30,0 g of zinc sulphate ($ZnSO_4$) in water and dilute to 100 ml in a volumetric flask with water.

4.3.2. Potassium hexacyanoferrate (II) solution.

Dissolve 17,2 g of potassium hexacyanoferrate (II) trihydrate (K4Fe(CN)6.3H20) and dilute to 100 ml in a volumetric flask with water.

5. APPARATUS

5.1. Analytical balance.

5.2. Waterbath, thermostatically controlled at 37 ^{o}C ± 1 ^{o}C.

5.3. Comparator, with special disc containing standard colour glasses calibrated in µg p-nitrophenol per ml milk, and 2 x 25 mm cells.

6. PROCEDURE

Precautions:

1. After use, test tubes must be emptied, rinsed in water, washed in hot water containing an al kaline detergent, followed by thorough rinsing in clean hot tap water. Finally, they must be rinsed in water and dried before use.

   Pipettes must be thoroughly rinsed in clean cold tap water immediately after use, followed by rinsing in water and dried before use.

2. The test tube stoppers must be thoroughly rinsed in hot tap water immediately after use, followed by boiling for two minutes in water.

3. The buffer substrate solution (4.2) should remain stable for at least one month if stored in a refrigerator at 4 ^{o}C or less. Any instability is denoted by the formation of a yellow colour. Whilst the test is always read against a boiled product control containing the same buffer substrate solution, it is recommended that the solution should not be used if it gives a colour reading in excess of 10 µg when read in a 25 mm cell in the comparator using distilled water in the other 25 mm cell.

4. Use a separate pipette for each sample and avoid contaminating the pipette with saliva.

5. The test must not be exposed to direct sunlight at any time.

6.1. Preparation of sample

Dissolve 10 g of the powder in 90 ml of water. The temperature for dissolving the powder must not exceed 35 ^{o}C.

6.2. Determination

6.2.1. Pipette 15 ml of buffer substrate (4.2) into a clean, dry test tube, followed by 2 ml of the reconstituted sample (6.1) to be tested. Stopper the tube, mix by inversion and place in the 37 ^{o}C water bath (5.2).

6.2.2. At the same time, place in the water bath a control tube containing 15 ml of buffer substrate and 2 ml of boiled reconstituted sample similar to that under test.

6.2.3. After two hours remove both tubes from the water bath, add 0,5 ml of zinc sulphate precipitant (4.3.1), replace the stopper, shake vigorously and allow to stand for three minutes. Add 0,5 ml of potassium hexacyanoferrate (II) precipitant (4.3.2), mix thoroughly and filter through the fluted filter paper (5.4) and collect the clear filtrate in the clean test tube.

6.2.4. Transfer the filtrate to a 25 mm cell and compare against the filtrate of the boiled sample control in the comparator using the special disc (5.3).

7. EXPRESSION OF RESULTS

7.1. Calculation

The direct reading obtained under 6.2.4 is recorded as µg p-nitrophenol per ml sample or per ml of reconstituted sample.

7.2. Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 2 µg of p-nitrophenol liberated by 1 ml of reconstituted milk.