I

(Acts whose publication is obligatory)

COMMISSION REGULATION (EEC) No 2188/81

of 28 July 1981

amending Regulation (EEC) No 625/78 on detailed rules of application for public storage of skimmed-milk powder

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Regulation (EEC) No 804/68 of 27 June 1978 on the common organization of the market in milk and milk products (1), as last amended by the Act of Accession of Greece, and in particular Article 7 (5) thereof,

Whereas Annex I to Commission Regulation (EEC) No 625/78 (2), as last amended by Regulation (EEC) No 2937/80 (3), lays down quality conditions which skimmed-milk powder must meet in order to be offered to intervention and contains certain requirements relating to the analytical procedures for detecting whey by determining the proportions of certain components in the skimmed-milk powder; whereas footnote (2) to the said Annex made provision for fixing Community limits for the said components following a certain experimental period within the Member States;

Whereas it is now possible to establish a method of analysis at Community level for determination of the whey rennet in the skimmed-milk powder by measuring the free sialic acid; whereas, however, this method of checking should be made obligatory only after a certain period within which the supervisory authorities and producers can familiarize themselves with this analysis procedure;

Whereas the measures provided for in this Regulation are in accordance with the opinion of the Management Committee for Milk and Milk Products,

HAS ADOPTED THIS REGULATION:

Article 1

1. Annex I to Regulation (EEC) No 625/78 is hereby amended as follows:

(a) in point 2 (b), the text of the second indent is replaced by the following:

'— whey:

determination of free sialic acid (2) and/or of the complex cysteine-cystine in cases where at least one of the three compulsory tests, i.e. the test for the whey glycomacropeptides (determined by a simplified test), the test for the lactates content or the test for the ash content exceeds 3 %, 150 mg/100 g and 8 % respectively';

(b) the text of footnote (2) is replaced by the following:

'(2) With regard to detection of whey rennet by measurement of free sialic acid, the method of analysis applicable as from 1 January 1982 shall be that fixed in Annex IV.'

2. The Annex to this Regulation is added as Annex IV to Regulation (EEC) No 625/78.

Article 2

This Regulation shall enter into force on the third day following its publication in the *Official Journal of the European Communities.*

(1) OJ No L 148, 28. 6. 1968, p. 13.
(2) OJ No L 84, 31. 3. 1978, p. 19.
(3) OJ No L 305, 14. 11. 1980, p. 13.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 28 July 1981.

For the Commission

The President

Gaston THORN

ANNEX

'ANNEX IV

DETECTION OF RENNET WHEY IN SKIMMED-MILK POWDER INTENDED FOR PUBLIC STORAGE BY QUANTITATIVE DETERMINATION OF FREE SIALIC ACID

1. **Scope and field of application**

This method allows the determination of rennet whey in skimmed-milk powder intended for public storage.

2. **Principle of the method**

When milk is coagulated as by renneting, some glycomacropeptides of casein, with a high content of a sialic acid, are freed. The dosage of sialic acid (N-acetylneuraminic acid) allows the detection of rennet whey eventually present in skimmed-milk powder. After reconstitution of milk powder, glycomacropeptides, including sialic acid, are removed from a filtrate containing 12 % trichloroacetic acid by precipitation with phosphotungstic acid.

Sialic acid, freed by acid hydrolysis, forms, with resorcinol, a coloured complex measured by visible spectrophotometry at 580 nm.

3. **Reagents**

All reagents shall be of analytical grade. The water used shall be distilled or deionized.

3.1. Trichloroacetic acid solution (TCA)

Dissolve 240 g of trichloroacetic acid in water and dilute to 1 000 ml.

3.2. Phosphotungstic acid solution

Dissolve 20 g of phosphotungstic acid in water and dilute to 100 ml.

3.3. Etahnol 95 % (v/v)

3.4. Sulphuric acid solution, about 0·1 N

Dilute 28·1 ml of concentrated sulphuric acid (95 % H_2SO_4) with water to 1 000 ml in order to obtain 1 N sulphuric acid solution. Dilute 100 ml of 1 N sulphuric acid solution with water to 1 000 ml in order to obtain 0·1 N sulphuric acid solution.

3.5. Acetate buffer, pH = 4·8

Dissolve 19·7 g of anhydrous sodium acetate in water, add 9 ml of glacial acetic acid and dilute with water to 1 000 ml. Check and if necessary adjust the pH value.

3.6. Copper sulphate solution, 0·1 M

Dissolve 2·497 g of copper sulphate ($CuSO_4 \cdot 5H_2O$) in water and dilute to 100 ml.

3.7. Resorcine solution, 2 %

Dissolve 2 g of resorcinol in water and dilute to 100 ml. This solution can be kept for four months at 5 to 8 °C.

3.8. Resorcinol reagent

Mix 10 ml of the resorcine solution (3.7), 80 ml of concentrated hydrochloric acid ($d_{20\,°C}$ = 1·19 g/l) and 0·25 ml copper sulphate solution (3.6) and dilute to 100 ml with water. Prepare fresh solution on the day of the use.

3.9. Iso-amyl-alcohol

3.10 N-acetyl-neuraminic acid (sialic acid) solution

Dissolve 20 mg of N-acetyl-neuraminic acid in water and dilute to 100 ml. This solution must not be kept longer than one week at + 4 °C.

4. **Apparatus**

4.1. Analytical balance

4.2. Magnetic stirrer, with stirring bars of 2 cm, teflon coated

4.3. pH meter

4.4. Centrifuge with a centrifugal force of 3 000 g

4.5. Centrifuge tubes of about 75-ml capacity

4.6. Waterbath, thermostatically controlled at 80 °C

4.7. Boiling waterbath

4.8. Filter papers, diameter 125 mm (Schleicher und Schüll, No 589[2], type "Weissband", or filters of equivalent efficiency)

4.9. Glass funnels, diameter of about 7 cm

4.10 Glass beakers of 100 and 150 ml

4.11. Volumetric flasks of 50, 100 and 1 000-ml capacity

4.12. Reagent tubes with screw caps of 10-ml capacity

4.13. Spectrophotometer

4.14. Photometer cells of glass (4.13) with 10-mm optical path length

4.15. Graduated pipettes of 1, 2, 5, 10, 20, 25 and 50-ml capacity

4.16. Glass stirring rods

4.17. Drying oven, thermostatically controlled at 35 °C.

5. **Preparation of the sample**

After homogenization, the sample is examined in accordance with the instructions given under paragraph 6 and there on.

6. **Procedure**

6.1. Isolation of free sialic acid

6.1.1. Weigh 5 ± 0·005 g of skimmed-milk powder into a glass beaker of 150 ml. After the addition of a magnetic stirrer bar (4.2) the sample is completely dissolved by stirring in 50 ml of water.

Add, by stirring, 50 ml of TCA solution (3.1). Allow to stand for about 30 minutes (at room temperature).

6.1.2. Stir and filter immediately (4.8).

6.1.3. Introduce into a centrifuge tube (4.5) 50 ml of the filtrate taken 60 minutes after the commencement of filtration.

1 ml of phosphotungstic acid solution (3.2) is added. Make the solution homogenous by stirring. After standing for 10 minutes at room temperature, the precipitate is separated by centrifugation for 10 minutes at 3 000 g.

6.1.4. The supernatant liquid is discarded. The sediment is washed twice with 5 ml of ethanol (3.3). Take care to mix well with a magnetic stirrer or a glass rod (4.16). (If a glass rod is used, introduce 2 ml of ethanol to disperse the precipitate, washing the glass rod with the remaining 3 ml of ethanol.)

After each treatment, centrifuge for 10 minutes at 3 000 g. The sediment is dried either overnight at room temperature or at at 35 °C for 90 minutes.

6.1.5. Add to the dried sediment 4 ml of 0·1 N sulphuric acid (3.4), mix thoroughly to disperse it. Close the containers and place them in a water bath at 80 °C for 40 minutes, stirring from time to time to ensure complete dispersion.

After cooling to room temperature, add 4 ml of acetate buffer (3.5) and again mix carefully.

6.1.6. Centrifuge for 10 minutes at 3 000 g. The determination of sialic acid or N-acetyl-neuraminic acid is carried out on the supernatant liquid.

6.2. Spectrophotometric determination of free sialic acid

6.2.1. Put 2 ml of the supernatant into a reagent tube (4.12) and add 2 ml resorcinol reagent (3.8).

6.2.2. Close the tube and mix carefully. Place the tubes in a water bath at 100 °C for exactly 15 minutes (4.7), followed by cooling to room temperature in running water.

6.2.3. Add 5 ml of iso-amyl-alcohol (3.9). Close the tubes, mix by vigorous shaking and place them in ice-water for 15 minutes.

6.2.4. Centrifuge the tubes for 2 minutes at 1 000 g in order to clarify both layers.

6.2.5. Transfer about 3 ml of the upper layer (iso-amyl-alcohol layer) into a glass optical cell and measure the extinction at 580 nm against the blank sample not later than 30 minutes after removing the tubes from the ice-water.

6.3. Blank test

Prepare a blank sample as described in 6.2.1 to 6.2.5 with the use of 1 ml 0·1 N sulphuric acid (3.4) and 1 ml acetate buffer (3.5) instead of 2 ml filtrate as described under 6.2.1.

6.4. Calibration curve

6.4.1. Introduce precisely 0-2-5-10-20 and 30 ml, corresponding to 0-0·4-1-2-4-6 mg sialic acid, of the solution (3.10) into 50-ml volumetric flasks; dilute to 50 ml with 0·1 N (3·4) sulphuric acid, mix by vigorous shaking.

6.4.2. 1 ml solution of each volumetric flask is mixed in a test tube (4.12) with 1 ml of acetate buffer (3.5), in order to obtain a range of standard solutions containing 0 (0 value) 8, 20, 40, 80, 120 micrograms of sialic acid respectively.

Mix carefully and follow the procedure according to 6.2.2 to 6.2.5.

6.4.3. In order to establish a calibration curve, prepare a graph of extinctions obtained as under 6.4.2 versus the corresponding sialic acid concentrations in micorgrams according to 6.4.2.

7. **Expression of the results**

7.1. Calculation

Calculate the sialic acid content expressed as micrograms per gram using the following formula:

$$\frac{C \cdot 8}{E}$$

where C is the mass of sialic acid in micrograms, evaluated from the calibration curve according to the extinction, obtained under 6.2.5 and E is the amount of the sample (6.1.1) in grams. Report the results to the nearest one microgram.

7.2. Repeatability

The difference between the results of a determination in duplicate achieved almost simultaneously by or in rapid succession by the same analyst shall not exceed 5 % of the arithmetic mean of the results.

8. **Interpretation of the results**

This method enables the presence of whey in the skimmed-milk powder to be quantified.

8.1. Calculation of the average value of the free sialic acid in micrograms per gram of the sample related to its content of protein expressed in percentage (m/m):

$$Y = 40 + 3\,(X - 34)^2$$

where:

40 = the average content of sialic acid in micrograms per gram in respect of a skimmed-milk powder containing at least 34 % protein,

X = total protein content (%) determined by Kjeldahl Procedure FIL:IDF 20:1962.

8.2. Calculation of percentage of whey powder which may be present:

$$\frac{Z - Y}{10}$$

where:

Z = the content in sialic acid of the sample as determined in 7.1 expressed in micrograms per gram,

Y = the average content of free sialic acid in the sample as calculated in 8.1,

10 = by convention the free sialic acid content expressed in micrograms of one gram of rennet whey powder.

8.3. Allowing for errors in the method and natural variations in the composition of the sample it may be concluded that whey is absent when the value obtained in 8.2 is 2·0 or less.

In cases where higher values are obtained the presence of whey is confirmed and is quantified according to the formula given in 8.2.'