COMMISSION REGULATION (EEC) No 2426/90

of 21 August 1990

amending Regulation (EEC) No 1725/79 on the rules for granting aid to skimmed milk processed into compound feedingstuffs and skimmed-milk powder intended in particular for feed for calves

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 1968 on the common organization of the market in milk and milk products (1), as last amended by Regulation (EEC) No 3879/89 (2), and in particular Article 10 (3) thereof,

Whereas Article 1 (2a) of Commission Regulation (EEC) No 1725/79 (3) as last amended by Regulation (EEC) No 3368/88 (4) states that skimmed-milk powder in the meaning of Article 1 (1) has to meet the definitions given in Article 1 of Council Regulation (EEC) No 986/68 (5), as last amended by Regulation (EEC) No 1115/89 (6), and has to be produced without any addition;

Whereas the fraudulent addition of whey solids to the skimmed milk used for the production of skimmed-milk powder or to the powder itself is contrary to the aforesaid provisions; whereas, in the absence of an official Community method for the detection of whey powder in skimmed-milk powder which may contain buttermilk powder, Regulation (EEC) No 1725/79 does not provide for specific rules on the detection of whey solids; whereas an analysis method has recently been developed for the detection of rennet whey; whereas it is appropriate to impose this method in the framework of the aforesaid Regulation;

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

Regulation (EEC) No 1725/79 is hereby amended as follows:

1. The following is added to the first subparagraph of Article 10 (3):

   'Where such inspections relate to skimmed-milk powder to be used, whether as such or in the form of a mixture, the absence of rennet whey powder is proven proven the procedure outlined in Annex IV.'

2. Annex I A (2) (j) is replaced by the following:

   '(j) others and especially acid whey as far as its detection is required by the national authorities.'

3. The Annex to this Regulation is added as Annex IV.

Article 2

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

It shall apply from 1 March 1991.

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

Done at Brussels, 21 August 1990.

For the Commission

Ray MAC SHARRY

Member of the Commission

(1) OJ No L 148, 28. 6. 1968, p. 13.
(2) OJ No L 378, 27. 12. 1989, p. 1.
(3) OJ No L 199, 7. 8. 1979, p. 1.
(4) OJ No L 296, 29. 10. 1988, p. 50.
(5) OJ No L 169, 18. 7. 1968, p. 4.
(6) OJ No L 118, 29. 4. 1989, p. 7.

ANNEX

'ANNEX IV

DETERMINATION OF RENNET WHEY SOLIDS IN SKIMMED-MILK POWDER AND MIXTURES UNDER REGULATION (EEC) No 1725/79

1. **Scope :** Detection of the addition of rennet whey solids to

(a) skimmed-milk powder as defined in Article 1 of Regulation (EEC) No 986/68, and

(b) mixtures as defined in Article 1 (3) of Regulation (EEC) No 1725/79.

2. **References :** International Standard ISO 707

Milk and milk products : — methods of sampling, conforming to the guidelines contained in Annex I (2) (c) to Regulation (EEC) No 625/78.

3. **Definition**

The content of rennet whey solids is defined as the percentage by mass as determined by the procedure described.

4. **Principle**

Determination of the amount of glycomacropeptide A pursuant to Annex V to Regulation (EEC) No 625/78. Samples giving positive results are analyzed for glycomacropeptide A by a reversed-phase high-performance liquid chromatography procedure (HPLC-procedure). Evaluation of the result obtained by reference to standard samples consisting of skimmed-milk powder with and without a known percentage of whey powder. Results higher than 1 % (m/m) show that rennet whey solids are present.

5. **Reagents**

All reagents must be of recognized analytical grade. The water used must be distilled water or water of at least equivalent purity. Acetonitrile should be of spectroscopic or HPLC quality.

Reagents needed for the procedure described in Regulation (EEC) No 625/78 are described in Annex V to that Regulation.

Reagents for reversed-phase HPLC.

5.1. *Trichloroacetic acid solution*

Dissolve 240 g of trichloroacetic acid (CCI_3COOH) in water and make up to 1 000 ml.

5.2. *Eluent A and B*

Eluent A : 150 ml of acetonitrile (CH_3CN), 20 ml of isopropanol ($CH_3CHOHCH_3$) and 1,00 ml trifluoroacetic acid (TFA, CF_3COOH) are made up with water to 1 000 ml. Eluent B : 550 ml of acetonitrile, 20 ml of isopropanol and 1,00 ml TFA are made up with water to 1 000 ml. Filter the eluent solution, prior to use, through a membrane filter with a 0,45 µm pore diameter.

5.3. *Conservation of the column*

After the analyses the column is flushed with eluent B (via a gradient) and subsequently flushed with acetonitrile (via a gradient in 30 minutes.). The column is stored in acetonitrile.

5.4. *Standard samples*

5.4.1. Skimmed-milk powder meeting the requirements of Regulation (EEC) No 625/78 (i. e. [0]).

5.4.2. The same skimmed-milk powder adulterated with 5 % (m/m) rennet-type whey powder of standard composition (i. e. [5]).

5.4.3. The same skimmed-milk powder adulterated with 50 % (m/m) rennet-type whey powder of standard composition (i. e. [50]) (*).

6. **Apparatus**

Apparatus needed for the procedure described in Regulation (EEC) No 625/78 is described in Annex V to that Regulation.

Apparatus for reversed-phase HPLC.

(*) Rennet-type whey powder of standard composition and also the adulterated skimmed-milk powder are available from NIZO, Kernhemseweg 2, PO Box 20 — NL-6710 BA, Ede.
However, powders giving equivalent results to the NIZO powders must also be used.

6.1. Analytical balance.

6.2. Centrifuge, capable of attaining a centrifugal force of 2 200 g, fitted with stoppered centrifuge tubes of about 50 ml.

6.3. Mechanical shaker with a provision to shake at 50 °C.

6.4. Magnetic stirrer.

6.5. Glass funnels, diameter about 7 cm.

6.6. Filter papers, medium filtration, diameter about 12,5 cm.

6.7. Glass filtration equipment with 0,45 µm pore diameter membrane filter.

6.8. Graduated pipettes, allowing delivery of 10 ml (ISO 648, Class A, or ISO/R 835), or a system capable of deliverying 10,0 ml in two minutes.

6.9. Thermostatic waterbath, set at 25 ± 0,5 °C.

6.10. HPLC-equipment, consisting of:

6.10.1. Binary gradient pumping system.

6.10.2. Injector, hand or automatic, with a 100 µl capacity.

6.10.3. Column Dupont Protein Plus (25 × 0,46 cm I.D.) or an equivalent wide-pore silica based reversed-phase column.

6.10.4. Thermostatic column oven, set at 35 ± 1 °C.

6.10.5. Variable wavelength UV detector, permitting measurements at 210 nm (if necessary, a higher wavelength up to 220 nm may be used) with a sensitivity of 0,02 Å.

6.10.6. Integrator capable of peak height measurement.

Note

Operation of the column at room temperature is possible, provided that the room temperature does not fluctuate more than 1 °C, otherwise too much variation in the retention time of GMP_A takes place.

7. Sampling

7.1. International standard ISO 707 — Milk and milk products — Methods of sampling, conforming to the guidelines contained in Annex I (2) (c) of Regulation (EEC) No 625/78.

7.2. Store the sample in conditions which preclude any deterioration or change in composition.

8. Procedure

8.1. *Preparation of the test sample*

Transfer the powder into a container with a capacity of about twice the volume of the powder, fitted with an airtight lid. Close the container immediately. Mix the milk powder well by means of repeated inversion of the container.

8.2. *Test portion*

Weigh 2,000 ± 0,001 g of test sample into a centrifuge tube (6.2) or suitable stoppered flask (50 ml).

8.3. *Removal of fat and proteins*

8.3.1. Add 20,0 g of warm water (50 °C) to the test portion. Dissolve the powder by shaking for five minutes or 30 minutes in the case of acid buttermilk using a mechanical shaker (6.3). Place the tube into the waterbath (6.9) and allow to equilibrate to 25 °C.

8.3.2. Add 10.0 ml of the trichloroacetic acid solution of 25 °C (5.1) constantly over two minutes, while stirring vigorously with the aid of the magnetic stirrer (6.4). Place the tube in a waterbath (6.9) and leave for 60 minutes.

8.3.3. Centrifuge (6.2) for 10 minutes at 2 200 g, or filter through paper (6.6), discarding the first 5 ml of filtrate.

8.4. *Chromatographic determination*

8.4.1. Perform HPLC-analysis as described in Regulation (EEC) No 625/78, Annex V. If a negative result is obtained, the sample analyzed does not contain rennet-whey solids in detectable amounts. In case of positive results the reversed-phase HPLC-procedure described below has to be applied. The presence of acid buttermilk powder may give rise to false-positive results. The reversed-phase HPLC method excludes this possibility.

8.4.2. Before the reversed phase HPLC-analysis is carried out, the gradient conditions should be optimized. A retention time of 26 ± 2 minutes for GMP_A is optimal for gradient systems with a dead volume of about 6 ml (volume from the point where the solvents come together to the volume of the injector loop, inclusive). Gradient systems with a lower dead volume (e. g. 2 ml) should used 22 minutes as an optimal retention time.

Take solutions of the standard samples (5.4) without and with 50 % rennet whey.

Inject 100 µl of supernatant or filtrate (8.3.3) into the HPLC apparatus operating at the scouting gradient conditions given in Table 1.

Table 1. *Scouting gradient conditions for optimization of the chromatography.*

Time (minutes)	Flow (ml/minutes)	% A	% B	Curve
Init	1,0	90	10	*
27	1,0	60	40	lin
32	1,0	10	90	lin
37	1,0	10	90	lin
42	1,0	90	10	lin

Comparison of the two chromatograms should reveal the location of the peak of GMP_A.

Using the formula given below, the initial solvent composition to be used for the normal gradient (see 8.4.3) can be calculated :

$$\% B = 10 - 2{,}5 + (13{,}5 + (RTgmpA - 26)/6)*30/27$$

$$\% B = 7{,}5 + (13{,}5 + (RTgmpA - 26)/6)*1.11$$

Where :

RTgmpA : retention time of GMP_A in the scouting gradient.

10 : the initial % B of the scouting gradient.

2,5 : % B at midpoint minus % B at initial in the normal gradient.

13,5 : midpoint time of the scouting gradient.

26 : required retention time of GMP_A.

6 : ratio of slopes of the scouting and normal gradient.

30 : % B at initial minus % B at 27 minutes in the scouting gradient.

27 : run-time of the scouting gradient.

8.4.3. Take solutions of the test samples.

Inject 100 µl of accurately measures supernatant or filtrate (8.3.3) into the HPLC apparatus operating at a flow rate of 1,0 ml of eluent solution (5.2) per minutes.

The composition of the eluent of the start of the analysis is obtained from 8.4.2. It is normally close to A : B = 76 :24 (5.2). Immediately after the injection a linear gradient is started, which results in a 5 % higher percentage of B after 27 minutes. Subsequently a linear gradient is started, which brings the eluent composition to 90 % B in five minutes. This composition is maintained for five minutes, after which the composition is changed, via a linear gradient in five minustes to the initial composition. Depending on the internal volume of the pumping system, the next injection can be made 15 minutes after reaching the initial conditions.

Remarks

1. The retention time of the glycomacropeptide should be 26 ± two minutes. This can be achieved by varying the initial and end conditions of the first gradient. However, the difference in the % B for the initial and end conditions of the first gradient must remain 5 % B.

2. The eluents should be degassed sufficiently and should also remain degassed. This is essential for proper functioning of the gradient pumping system. The standard deviation for the retention time of the GMP peak should be smaller than 0,1 minutes ($n = 10$).

3. Every five samples the reference sample [5] should be injected and used to calculate a new response factor R (9.1.1).

8.4.4. The results of the chromatographic analysis of the test sample [E] are obtained in the form of a chromatogram in which the GMP peak is identified by its retention time of about 26 minutes.

The integrator (6.40.6) automatically calculates the peak height H of the GMP peak. The baseline location should be checked in every chromatogram. The analysis or the integration should be repeated if the baseline was incorrectly located.

It is essential to examine the appearance of each chromatogram prior to quantitative interpretation, in order to detect any abnormalities due either to malfunctioning of the apparatus or the column, or to the origin and nature of the sample analyzed. If in doubt, repeat the analysis.

8.5. *Calibration*

8.5.1. Apply exactly the procedure described from point 8.2 to point 8.4.4 to the standard samples (5.4.1 to 5.4.2). Use freshly prepared solutions, because GMP degrades in an 8 % trichloroacetic acid environment at room temperature. At 4 °C the solution remains stable for 24 hours. In the case of long series of analyses the use of a cooled sample tray in the automatic injector is desirable.

Note

8.4.2 may be omitted if the % B at initial conditions is known from previous analyses.

The chromatogram of the reference sample [5] should be analogous to Fig. 1. In this figure the GMP_A peak is preceded by two small peaks. It is essential to obtain a similar separation.

8.5.2. Prior to chromatographic determination of the samples inject 100 µl of the standard sample without rennet whey [0] (5.4.1).

The chromatogram should not show a peak at the retention time of the GMP_A peak.

8.5.3. Determine the response factors R by injecting the same volume of filtrate (8.5.1) as used for the samples.

9. Expression of results

9.1. *Method of calculation and formulae*

9.1.1. Calculation of the response factor R :

GMP peak : $R = W/H$

Where :

R = the response factor of the GMP peak.
H = the height of the GMP peak.
W = the quantity of whey in the standard sample [5].

9.2. *Calculation of the percentage of rennet whey powder in the sample :*

$W[E] = R \times H[E]$

Where :

W[E] = the percentage (m/m) of rennet whey in the sample [E].
R = the response factor of the GMP peak (9.1.1).
H[E] = the height of the GMP peak of the sample [E].

If W[E] is greater than 1 % and the difference between the retention time and that of the standard sample [5] is smaller than 0,2 minutes then rennet whey solids are present.

9.3. *Accuracy of the procedure*

9.3.1. Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst using the same apparatus on identical test material shall not exceed 0,2 % m/m.

9.3.2. Reproducibility

Not yet determined.

9.3.3. Linearity

From 0 to 16 % of rennet whey a linear relationship should be obtained with a coefficient of correlation > 0,99.

9.4. **Interpretation**

9.4.1. Whey is considered to be present if the result obtained in 9.2 is higher than 1 % m/m and the retention time of the GMP peak differs less than 0,2 minutes from that of the standard sample [5]. The 1 % limit is fixed in agreement with the provisions of points 9.2 and 9.4.1 of Annex V to Regulation (EEC) No 625/78.

Table 1 — Ni—4.6 standard

