Commission Implementing Regulation (EU) 2016/1240 of 18 May 2016 laying down rules for the application of Regulation (EU) No 1308/2013 of the European Parliament and of the Council with regard to public intervention and aid for private storage (Text with EEA relevance)

ANNEX V

SKIMMED MILK POWDER

[F1 Appendix III

DETERMINING RENNET WHEY SOLIDS IN SKIMMED MILK POWDER

1. PURPOSE: DETECTING THE ADDITION OF RENNET WHEY SOLIDS TO SKIMMED MILK POWDER

2. REFERENCES: INTERNATIONAL STANDARD ISO 707

3. DEFINITION

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

4. PRINCIPLE

Samples are analysed for caseinomacropeptide A by a reversed-phase high-performance liquid chromatography procedure (HPLC procedure). Evaluation of the result is 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 shall be of recognised analytical grade. The water used shall be distilled water or water of at least equivalent purity. Acetonitrile should be of spectroscopic or HPLC quality.

5.1. **Trichloroacetic acid solution**

Dissolve 240 g of trichloroacetic acid (CCl_3COOH) in water and make up to 1 000 ml. The solution should be clear and colourless.

5.2. **Eluents A and B**

Eluent A: 150 ml of acetonitrile (CH_3CN), 20 ml of isopropanol ($CH_3CHOHCH_3$), and 1,00 ml of trifluoroacetic acid (TFA, CF_3COOH) are placed in a 1 000 ml volumetric flask. Make up to 1 000 ml with water.

Eluent B: 550 ml of acetonitrile, 20 ml of isopropanol and 1,00 ml of TFA are placed in a 1 000 ml volumetric flask. Make up to 1 000 ml with water. 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 for 30 minutes). The column is stored in acetonitrile.

5.4. **Standard samples**

5.4.1. *Skimmed milk powder meeting the requirements for public storage (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

6.1. **Analytical balance**

6.2. **Optional centrifuge capable of attaining a centrifugal force of 2 200 g, fitted with stoppered or capped centrifuge tubes of about 50 ml capacity**

6.3. **Mechanical shaker**

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 dispensing system capable of delivering 10,0 ml in two minutes**

6.9. **Dispensing system capable of delivering 20,0 ml water at ca. 50 °C**

6.10. **Thermostatic water bath, set at 25 ± 0,5 °C**

6.11. **HPLC equipment, consisting of:**

6.11.1. *Binary gradient pumping system*

6.11.2. *Injector, hand or automatic, with a 100 µl capacity*

6.11.3. *Agilent Technologies Zorbax 300 SB-C3 column (length 25 cm, 0,46 cm internal diameter) or an equivalent wide-pore silica based reversed-phase column*

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

6.11.5. *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*

6.11.6. *Integrator capable of setting the integration to common baseline or valley-to-valley*

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 CMP_A takes place.

7. SAMPLING

7.1. **Samples shall be taken in accordance with the procedure laid down in International Standard ISO 707. However, [F2the appropriate authority] may use another method of sampling provided that it complies with the principles of the abovementioned standard**

Textual Amendments

F2 Words in Annex 5 Pt. 1A Appendix 3 para. 7.1 substituted (31.12.2020) by The Market Measures Payment Schemes (Amendment) (EU Exit) Regulations 2019 (S.I. 2019/823), regs. 1, **6(47)(a)(ii)**; 2020 c. 1, **Sch. 5 para. 1(1)**

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 milk 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 2,00 ± 0,001 g of test sample into a centrifuge tube (6.2) or suitable stoppered flask (50 ml).

Note: In the case of mixtures, weigh such an amount of the test sample that the defatted sample portion corresponds to 2,00 g.

8.3. **Removal of fat and proteins**

8.3.1. *Add 20,0 ml of warm water (50 °C) to the test portion. Dissolve the powder by shaking for five minutes using a mechanical shaker (6.3). Place the tube into the water bath (6.10) and allow to equilibrate to 25 °C*

8.3.2. *Add 10,0 ml of the trichloroacetic acid solution of ca. 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 water bath (6.10) and leave for 60 minutes*

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

8.4. **Chromatographic determination**

8.4.1. *The reversed-phase HPLC method excludes the possibility false-positive results due to the presence of acid buttermilk powder.*

8.4.2. *Before the reversed phase HPLC-analysis is carried out, the gradient conditions should be optimised. A retention time of 26 ± 2 minutes for CMP $_A$ is optimal for gradient systems having 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 having a lower dead volume (e.g. 2 ml) should use 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 optimisation of the chromatography

Time (min)	Flow (ml/min)	% A	% B	Curve
Initial	1,0	90	10	*
27	1,0	60	40	linear
32	1,0	10	90	linear
37	1,0	10	90	linear

42	1,0	90	10	linear

Comparison of the two chromatograms should reveal the location of the peak of CMP_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 + (RT_{cmpA} – 26) / 6) * 30 / 27 % B = 7,5 + (13,5 + (RT_{cmpA} – 26) / 6) * 1,11

Where:

RT_{cmpA}	:	retention time of CMP_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 CMP_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 measured 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 minute.

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 minutes 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.

Note 1. The retention time of the CMP_A should be 26 ± 2 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 shall remain 5 % B.

Note 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 CMP_A peak should be smaller than 0,1 minutes (n = 10).

Note 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 CMP_A peak is identified by its retention time of about 26 minutes*

The integrator (6.11.6) automatically calculates the peak height H of the CMP_A peak. The baseline location should be checked in every chromatogram. The analysis or the integration should be repeated if the baseline was incorrectly located.

Note: If the CMP_A peak is sufficiently separated from other peaks valley-to-valley baseline allocation should be used, otherwise use dropping perpendiculars to a common baseline, which should have starting point close to the CMP_A peak (thus not at t = 0 min!).Use for the standard and the samples the same type integration type and check in case of common baseline its consistency for the samples and the standard.

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 analysed. 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 CMP 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 Figure. 1. In this figure the CMP_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 CMP_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:*

CMP_A peak: $R = W/H$

Where:

R	=	the response factor of the CMP_A peak
H	=	the height of the CMP_A 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 CMP_A peak (9.1.1)
H(E)	=	the height of the CMP_A 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 determined.

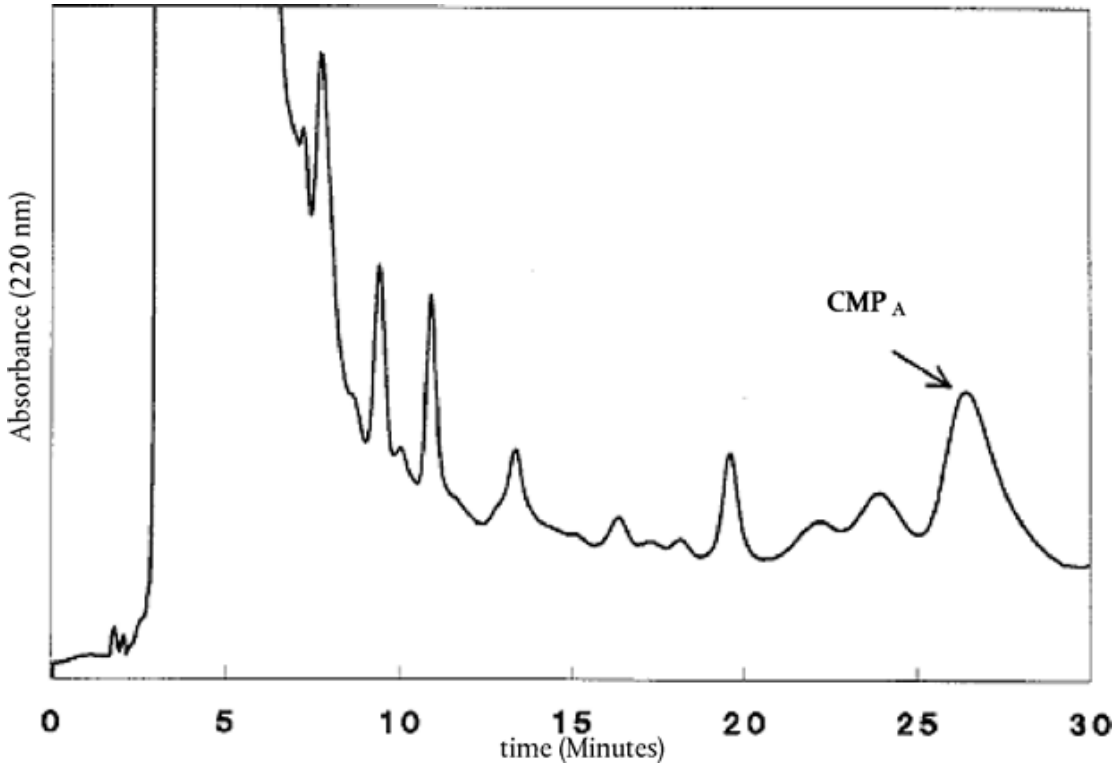
9.3.3. *Linearity*

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

9.4. **Interpretation**

The 1 % limit includes the uncertainty due to reproducibility.

Figure 1 **Ni—4.6 standard]**



Changes to legislation:
There are currently no known outstanding effects for the Commission Implementing Regulation (EU) 2016/1240, Appendix III .