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Status: *Point in time view as at 31/01/2020.*
Changes to legislation: *There are currently no known outstanding effects for the Commission*
Implementing Regulation (EU) 2016/1240, Appendix II. (See end of Document for details)
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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 II

DETECTION OF RENNET WHEY IN SKIMMED MILK POWDER FOR PUBLIC STORAGE BY DETERMINATION OF CASEINOMACROPEPTIDES HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.   SCOPE AND FIELD OF APPLICATION

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

2.   REFERENCE

International Standard ISO 707 - Milk and Milk Products - Guidance on sampling.

3.   DEFINITION

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

4.   PRINCIPLE
—   Reconstitution of the skimmed milk powder, removal of fat and proteins with trichloroacetic acid, followed by centrifugation or filtration;
—   Determination of the quantity of caseinomacropeptides (CMP) in the supernatant by high-performance liquid chromatography (HPLC);
—   Evaluation of the result obtained for the samples by reference to standard samples consisting of skimmed milk powder with or without the addition of a known percentage of whey powder.

5.   REAGENTS

All reagents shall be of recognised analytical grade. The water used shall be distilled water or water of at least equivalent purity.

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.   **Eluent solution, pH 6,0**

Dissolve 1,74 g of dipotassium hydrogen phosphate (K_2HPO_4), 12,37 g of potassium dihydrogen phosphate (KH_2PO_4) and 21,41 g of sodium sulphate (Na_2SO_4) in about 700 ml of water. Adjust, if necessary, to pH 6,0, using a solution of phosphoric acid or potassium hydroxide.

Make up to 1 000 ml with water and homogenise.

Note: The composition of the eluent can be updated to comply with the certificate of the standards or the recommendations of the manufacturer of the column packing material.

Filter the eluent solution, prior to use, through a membrane filter with a 0,45 μm pore diameter.

5.3.   **Flushing solvent**

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Mix one volume acetonitrile (CH_3CN) with nine volumes water. Filter the mixture prior to use through a membrane filter with a 0,45 μm pore diameter.

Note: Any other flushing solvent with a bactericidal effect which does not impair the columns' resolution efficiency may be used.

5.4.   **Standard samples**

5.4.1.   *Skimmed milk powder meeting the requirements of this Regulation (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])*

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.   *Pump*

6.11.2.   *Injector, hand or automatic, with a 15 to 30 μl capacity*

6.11.3.   *Two TSK 2 000-SW columns in series (length 30 cm, internal diameter 0,75 cm) or equivalent columns (e.g. single TSK 2 000-SWxl, single Agilent Technologies Zorbax GF 250) and a precolumn (3 cm × 0,3 cm) packed with I 125 or material of equivalent effectiveness*

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

6.11.5.   *Variable wavelength UV detector, permitting measurements at 205 nm with a sensitivity of 0,008 Å*

6.11.6.   *Integrator capable of valley-to-valley integration*

Note: Working with columns kept at room temperature is possible, but their power of resolution is slightly lower. In that case, the temperature should vary by less than ± 5 °C in any one range of analyses.

7.   SAMPLING

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

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**

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

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 (5.1) of ca. 25 °C in 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) 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.   *Inject 15 to 30 μl of accurately measured supernatant or filtrate (8.3.3) into the HPLC apparatus (6.11) operating at a flow rate of 1,0 ml of eluent solution (5.2) per minute*

Note 1. Another flow rate may be used, dependent of the internal diameter of the columns used or the instructions of the manufacturer of the column.

Note 2. Rinse the columns with water during each interruption. Never leave the eluent solution in them (5.2).

Prior to any interruption of more than 24 hours, rinse the columns with water then wash them with solution (5.3) for at least three hours at a flow rate of 0,2 ml per minute.

8.4.2.   *The results of chromatographic analysis of the test sample [E] are obtained in the form of chromatogram in which each peak is identified by its retention time RT as follows:*

Peak II:	The second peak of the chromatogram having an RT of about 12,5 minutes.
Peak III:	The third peak of the chromatogram, corresponding to the CMP, having an RT of 15,5 minutes.

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The choice of the column(s) can affect the retention times of the individual peaks considerably.

The integrator (6.11.6) automatically calculates the area A of each peak:

A_{II}:	area of peak II,
A_{III}:	area of peak III,

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 columns, 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 8.4.2 to the standard samples (5.4)*

Use freshly prepared solutions, because CMP degrade in an 8 % trichloroacetic environment. The loss is estimated at 0,2 % per hour at 30 °C.

8.5.2.   *Prior to chromatographic determination of the samples, condition the columns by repeatedly injecting the standard sample (5.4.2) in solution (8.5.1) until the area and retention time of the peak corresponding to the CMP are constant*

8.5.3.   *Determine the response factors R by injecting the same volume of filtrates (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 factors R:*

Peak II:	$R_{II} = 100/(A_{II}[0])$

where:

R_{II} = the response factors of peaks II,
$A_{II}[0]$ = the areas of peaks II of the standard sample [0] obtained in 8.5.3.

Peak III:	$R_{III} = W/(A_{III}[5] - A_{III}[0])$

where:

R_{III} = the response factor of peak III,
$A_{III}[0]$ and $A_{III}[5]$ = the areas of peak III in standard samples [0] and [5] respectively obtained in 8.5.3,
W = the quantity of whey in standard sample [5], i.e. 5.

9.1.2.   *Calculation of the relative area of the peaks in the sample [E]*
$S_{II}[E] = R_{II} \times A_{II}[E]$
$S_{III}[E] = R_{III} \times A_{III}[E]$
$S_{IV}[E] = R_{IV} \times A_{IV}[E]$

where:

S_{II} [E], S_{III} [E], S_{IV} [E]   =   the relative areas of peaks II, III and IV respectively in the sample [E],

A_{II} [E], A_{III} [E]   =   the areas of peaks II and III respectively in the sample [E] obtained in 8.4.2,

R_{II}, R_{III} =   the response factors calculated in 9.1.1.

9.1.3.   *Calculation of the relative retention time of peak III in sample [E]:*

$RRT_{III}[E] = (RT_{III}[E])/(RT_{III}[5])$

where:

RRT_{III} [E]   =   the relative retention time of peak III in sample [E],
RT_{III} [E]   =   the retention time of peak III in sample [E] obtained in 8.4.2,
RT_{III} [5]   =   the retention time of peak III in control sample [5] obtained in 8.5.3.

9.1.4.   *Experiments have shown that there is a linear relation between the relative retention time of peak III, i.e. RRT_{III} [E] and the percentage of whey powder added up to 10 %*

—   The RRT_{III} [E] is < 1,000 when the whey content is > 5 %;
—   The RRT_{III} [E] is ≥ 1,000 when the whey content is ≤ 5 %.

The uncertainty allowed for the values of RRT_{III} is ± 0,002.

Normally the value of RRT_{III} [0] deviates little from 1,034. Depending on the condition of the columns, the value may approach 1,000, but it shall always be greater.

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

$W = S_{III}[E] – [1, 3 + (S_{III}[0] – 0,9)]$

where:

W   =   the percentage m/m of rennet whey in the sample [E];
S_{III} [E]   =   the relative area of peak III of test sample [E] obtained as in 9.1.2;
1,3   =   represents the relative average area of peak III expressed in grams of rennet whey per 100 g determined in non-adulterated skimmed milk powder of various origins. This figure was obtained experimentally;
S_{III} [0]   =   represents the relative area of peak III which is equal to $R_{III} \times A_{III}$ [0]. These values are obtained in 9.1.1 and 8.5.3 respectively;
$(S_{III} [0] – 0,9)$ =   represents the correction to be made to the relative average area 1,3 when S_{III} [0] is not equal to 0,9. Experimentally the relative average area of peak III of the control sample [0] is 0,9.

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*

The difference between two single and independent results, obtained in two different laboratories on identical test material shall not exceed 0,4 % m/m.

9.4. **Interpretation**

9.4.1. *Assume the absence of whey if the relative area of peak III, S_{III} [E] expressed in grams of rennet whey per 100 g of the product is $\leq 2,0 + (S_{III}[0] - 0,9)$*

where

2,0	is the maximum value allowed for the relative area of peak III taking into account the relative average area of peak III, i.e. 1,3, the uncertainty due to variations in the composition of skimmed milk powder and the reproducibility of the method (9.3.2),
$(S_{III} [0] - 0,9)$	is the correction to be made when the area $S_{III} [0]$ is different from 0,9 (see point 9.2)

9.4.2. *If the relative area of peak III, S_{III} [E] is $> 2,0 + (S_{III}[0] - 0,9)$ and the relative area of peak II, S_{II} [E] ≤ 160, determine the rennet whey content as indicated in point 9.2.*

9.4.3. *If the relative area of peak III, S_{III} [E] is $> 2,0 + (S_{III}[0] - 0,9)$ and the relative area of peak II, S_{II} [E] ≤ 160, determine the total protein content (P %); then examine graphs 1 and 2.*

9.4.3.1. *The data obtained after analysis of samples of unadulterated skimmed milk powders with a high total protein content have been assembled in graphs 1 and 2.*

The continuous line represents the linear regression, the coefficients of which are calculated by the least squares method.

The dashed straight line fixes the upper limit of the relative area of peak III with a probability of not being exceeded in 90 % of cases.

The equations for the dashed straight lines of graphs 1 and 2 are:

$S_{III} = 0,376$ P % $- 10,7$	(graph 1),
$S_{III} = 0,0123$ S_{II} [E] $+ 0,93$	(graph 2),

respectively where:

S_{III} is the relative area of peak III calculated either according to total protein content or according to the relative area of peak S_{II} [E],

P % is the total protein content expressed as a percentage, by weight,

S_{II} [E] is the relative area of sample calculated in point 9.1.2.

These equations are equivalent to the figure of 1,3 mentioned in point 9.2.

The discrepancy (T_1 and T_2) between the relative area S_{III} [E] found and the relative area S_{III} is given by means of the following: $T_1 = S_{III}[E] - [(0,376 \text{ P\%} - 10,7) + (S_{III}[0] - 0,9)] T_2 = S_{III}[E] - [(0,0123 \text{ } S_{II}[E] + 0,93) + (S_{III}[0] - 0,9)]$

9.4.3.2.

If T_1 and/or T_2	are zero or less, the presence of rennet whey cannot be determined.
If T_1 and T_2	exceed zero, rennet whey is present.

The rennet whey content is calculated according to the following formula: $W = T_2 + 0,91$
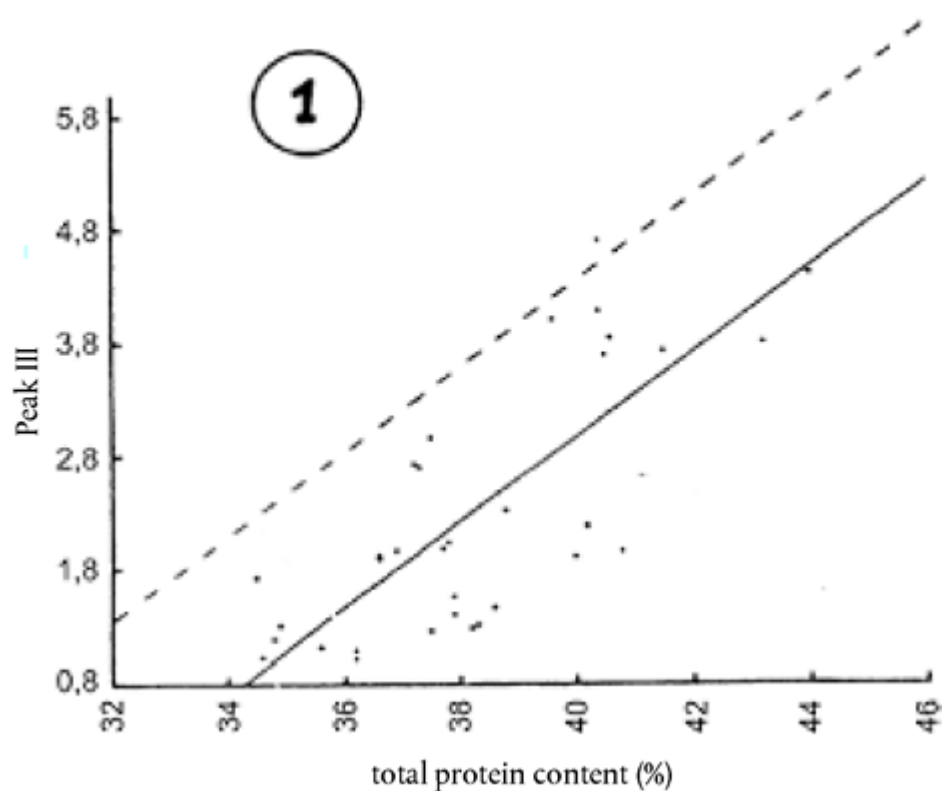
where:

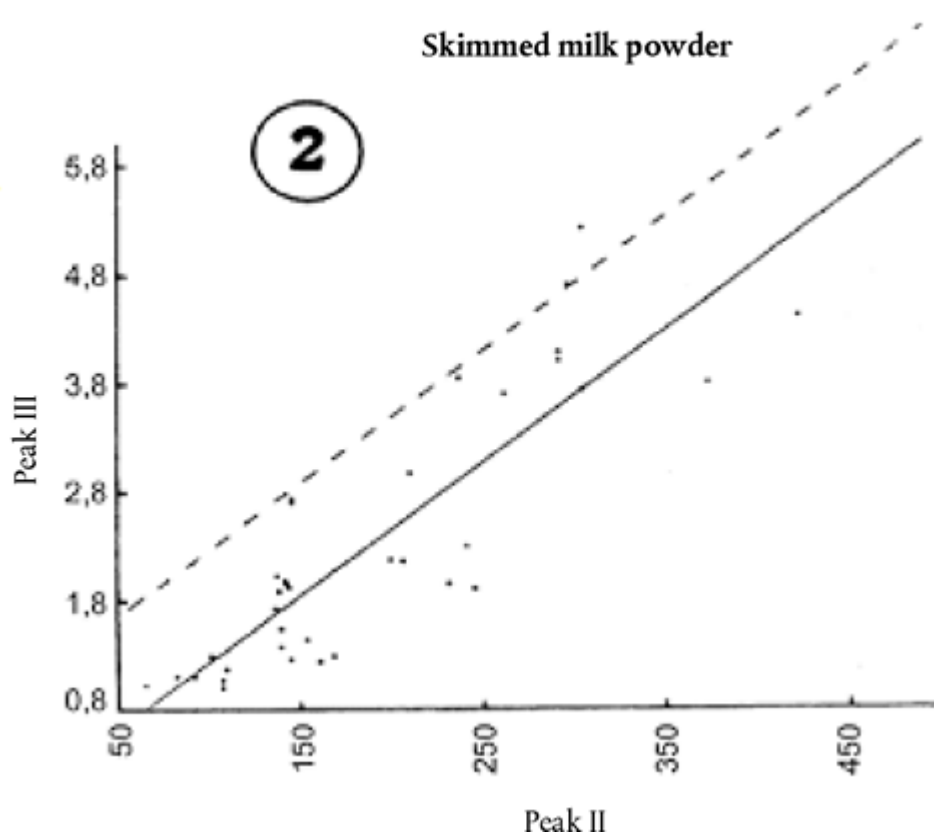0,91 is the distance on the vertical axis between the continuous and dotted straight lines.]

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