COMMISSION IMPLEMENTING REGULATION (EU) 2018/150

of 30 January 2018

amending Implementing Regulation (EU) 2016/1240 as regards methods for the analysis and quality evaluation of milk and milk products eligible for public intervention and aid for private storage

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EU) No 1306/2013 of the European Parliament and of the Council of 17 December 2013 on the financing, management and monitoring of the common agricultural policy and repealing Council Regulations (EEC) No 352/78, (EC) No 165/94, (EC) No 2799/98, (EC) No 814/2000, (EC) No 1290/2005 and (EC) No 485/2008 ([1]), and in particular Article 62(2)(i) thereof,

Whereas:

(1) Commission Delegated Regulation (EU) 2016/1238 ([2]) and Commission Implementing Regulation (EU) 2016/1240 ([3]) lay down the rules on public intervention and aid for private storage. Commission Regulation (EC) No 273/2008 ([4]) sets out the methods to be applied in assessing whether milk and milk products comply with the eligibility requirements laid down in those Regulations for public intervention and aid for private storage.

(2) In the light of technical developments in the methodology used in the analysis and quality evaluation of milk and milk products, substantial changes should be made in order to simplify and to provide for updated references to ISO standards. In the interests of clarity and efficiency, and having regard to the extent and technical nature of the amendments to the provisions of Regulation (EC) No 273/2008, the relevant provisions of that Regulation should be incorporated into Implementing Regulation (EU) 2016/1240.

(3) In order to ensure uniform compliance with the new standards and methods across Member States, laboratories should be allowed a sufficient period of time to review procedures and apply the updated methods.

(4) Implementing Regulation (EU) 2016/1240 should therefore be amended accordingly.

(5) In the interests of legal certainty Regulation (EC) No 273/2008 should be repealed.

(6) The measures provided for in this Regulation are in accordance with the opinion of the Committee for the Common Organisation of the Agricultural Markets,

HAS ADOPTED THIS REGULATION:

Article 1

Implementing Regulation (EU) 2016/1240 is amended as follows:

(1) Article 4 is amended as follows:

(a) paragraph 1 is amended as follows:

(i) point (d) is replaced by the following:

'(d) for butter: in Parts I and Ia of Annex IV to this Regulation';

(ii) point (e) is replaced by the following:

'(e) for skimmed milk powder: in Parts I and Ia of Annex V to this Regulation';

([1]) OJ L 347, 20.12.2013, p. 549.
([2]) Commission Delegated Regulation (EU) 2016/1238 of 18 May 2016 supplementing Regulation (EU) No 1308/2013 of the European Parliament and of the Council with regard to public intervention and aid for private storage (OJ L 206, 30.7.2016, p. 15).
([3]) 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 (OJ L 206, 30.7.2016, p. 71).
([4]) Commission Regulation (EC) No 273/2008 of 5 March 2008 laying down detailed rules for the application of Council Regulation (EC) No 1255/1999 as regards methods for the analysis and quality evaluation of milk and milk products (OJ L 88, 29.3.2008, p. 1).

(b) paragraph 2 is replaced by the following:

'2. The methods to be used to determine the quality of cereals, butter and skimmed milk powder eligible for public intervention referred to in Annexes I, IV and V respectively, shall be those established by the latest versions of the relevant European or international standards, as the case may be, in force at least 6 months before the first day of the public intervention period as defined in Article 12 of Regulation (EU) No 1308/2013.';

(2) the following Article 60a is inserted:

'*Article 60a*

Specific provision on checks relating to public intervention and aid for private storage for milk and milk products

1. The eligibility of butter, skimmed milk powder and cheese to receive aid for private storage shall be established in accordance with the methods laid down in Annexes VI, VII and VIII respectively.

Those methods shall be established by reference to the latest versions of the relevant European or international standards, as the case may be, in force at least 6 months before the first day of the public intervention period as defined in Article 12 of Regulation (EU) No 1308/2013.

2. The results of the checks conducted by applying the methods set out in this Regulation shall be evaluated in accordance with Annex IX.';

(3) the Annexes are amended in accordance with the Annex to this Regulation.

Article 2

Regulation (EC) No 273/2008 is repealed.

Article 3

This Regulation shall enter into force on the seventh day following that of its publication in the *Official Journal of the European Union*.

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

Done at Brussels, 30 January 2018.

For the Commission
The President
Jean-Claude JUNCKER

ANNEX

The Annexes to Implementing Regulation (EU) 2016/1240 are amended as follows:

(1) Annex IV is amended as follows:

(a) in Part I, point 2, the second sub-paragraph is replaced by the following:

'Each sample shall be assessed individually. No resampling or re-evaluation is allowed.';

(b) the following Part Ia is inserted:

'PART IA

Methods of analysis of unsalted butter for public intervention

Parameter	Method
Fat [1]	ISO 17189 or ISO 3727 part 3
Water	ISO 3727 part 1
Non-fat solids	ISO 3727 part 2
Fat acidity	ISO 1740
Peroxide value	ISO 3976
Non-milk fat	ISO 17678
Sensory characteristics	ISO 22935 parts 2 and 3 and scoring table hereafter.

[1] The method to be applied shall be approved by the paying agency.

Scoring table

Appearance		Consistency		Odour and Flavour	
Points	Remarks	Points	Remarks	Points	Remarks
5	*Very good* Ideal type Highest quality (equal dry)	5	*Very good* Ideal type Highest quality (equal spreadable)	5	*Very good* Ideal type Highest quality (absolutely pure finest odour)
4	*Good* (no evident defects)	4	*Good* (no evident defects)	4	*Good* (no evident defects)
1, 2 or 3	Any defect	1, 2 or 3	Any defect	1, 2 or 3	Any defect'

(2) in Annex V the following Part Ia is inserted:

'PART IA

Methods of analysis of skimmed milk powder for public intervention

Parameter	Method
Protein	ISO 8968 part 1
Fat	ISO 1736
Water	ISO 5537
Acidity	ISO 6091
Lactates	ISO 8069
Phosphatase test	ISO 11816 part 1
Insolubility index	ISO 8156
Scorched particles ([1])	ADPI
Micro-organisms	ISO 4833-part 1
Buttermilk	Appendix I
Rennet whey ([2])	Appendix II and III
Acid whey ([3])	ISO 8069 or On-the-spot inspections
Sensory checks ([4])	ISO 22935 part 2 and 3

([1]) Scorched particles' analyses may be conducted systematically. However, such analyses shall always be conducted if no sensory checks are performed.
([2]) The method to be applied shall be approved by the paying agency (one or both methods).
([3]) The method to be applied shall be approved by the paying agency.
([4]) Sensory checks shall be performed where deemed necessary after risk based analysis approved by the paying agency.

Appendix I

SKIMMED MILK POWDER: QUANTITATIVE DETERMINATION OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE

Method: reversed-phase HPLC

1. PURPOSE AND FIELD OF APPLICATION

The method describes a procedure for the quantitative determination of phosphatidylserine (PS) and phosphatidylethanolamine (PE) in skimmed milk powder (SMP) and is suitable for detecting buttermilk solids in SMP.

2. DEFINITION

PS + PE content: the mass fraction of substance determined using the procedure here specified. The result is expressed as milligrams of phosphatidylethanolamine dipalmitoyl (PEDP) per 100 g powder.

3. PRINCIPLE OF THE METHOD

Extraction of aminophospholipids by methanol from reconstituted milk powder. Determination of PS and PE as o-phthaldialdehyde (OPA) derivatives by reversed-phase (RP) HPLC and fluorescence detection. Quantification of PS and PE content in the test sample by reference to a standard sample containing a known amount of PEDP.

4. REAGENTS

All reagents shall be of recognised analytical grade. Water shall be distilled or water of at least equivalent purity, unless otherwise specified.

4.1. **Standard material: PEDP, at least 99 % pure**

Note: Standard material shall be stored at – 18 °C.

4.2. **Reagents for standard sample and test sample preparation**

4.2.1. *HPLC-grade methanol*

4.2.2. *HPLC-grade chloroform*

4.2.3. *Tryptamine-monohydrochloride*

4.3. **Reagents for o-phthaldialdehyde derivatisation**

4.3.1. *Sodium hydroxide, 12 M water solution*

4.3.2. *Boric acid, 0,4 M water solution adjusted to pH 10,0 with sodium hydroxide (4.3.1)*

4.3.3. *2-mercaptoethanol*

4.3.4. *o-phthaldialdehyde (OPA)*

4.4. **HPLC elution solvents**

4.4.1. *Elution solvents shall be prepared using HPLC-grade reagents.*

4.4.2. *HPLC-grade water*

4.4.3. *Methanol of tested fluorimetric purity*

4.4.4. *Tetrahydrofuran*

4.4.5. *Sodium dihydrogen phosphate*

4.4.6. *Sodium acetate*

4.4.7. *Acetic acid.*

5. APPARATUS

5.1. **Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg**

5.2. **Beakers, 25 and 100 ml capacity**

5.3. **Pipettes, capable of delivering 1 and 10 ml**

5.4. **Magnetic stirrer**

5.5. **Graduated pipettes, capable of delivering 0,2, 0,5 and 5 ml**

5.6. **Volumetric flasks, 10, 50 and 100 ml capacity**

5.7. **Syringes, 20 and 100 µl capacity**

5.8. **Ultrasonic bath**

5.9. **Centrifuge, capable of operating at 27 000 × g**

5.10. **Glass vials, about 5 ml capacity**

5.11. **Graduated cylinder, 25 ml capacity**

5.12. **pH-meter, accurate to 0,1 pH units**

5.13. **HPLC equipment**

5.13.1. *Gradient pumping system, capable of operating at 1,0 ml/min at 200 bar*

5.13.2. *Autosampler with derivatisation capability*

5.13.3. *Column heater, capable of maintaining the column at 30 °C ± 1 °C*

5.13.4. *Fluorescence detector, capable of operating at 330 nm excitation wavelength and 440 nm emission wavelength*

5.13.5. *Integrator or data processing software capable of peak area measurement*

5.13.6. *A LiChrospher® — 100 column (250 × 4,6 mm) or an equivalent column packed with octadecylsilane (C 18), 5 µm particle size.*

6. SAMPLING

Sampling shall be carried out in accordance with ISO Standard 707.

7. PROCEDURE

7.1. **Preparation of the internal standard solution**

7.1.1. *Weigh 30,0 ± 0,1 mg of tryptamine-monohydrochloride (4.2.3) into a 100 ml volumetric flask (5.6) and make up to the mark with methanol (4.2.1)*

7.1.2. *Pipette 1 ml (5.3) of this solution into a 10 ml volumetric flask (5.6) and make up to the mark with methanol (4.2.1) in order to obtain a 0,15 mM tryptamine concentration*

7.2. **Preparation of the test sample solution**

7.2.1. *Weigh 1,000 ± 0,001 g of the SMP sample into a 25 ml beaker (5.2). Add 10 ml of distilled water at 40 °C ± 1 °C by a pipette (5.3) and stir with a magnetic stirrer (5.4) for 30 minutes in order to dissolve any lumps*

7.2.2. *Pipette 0,2 ml (5.5) of the reconstituted milk into a 10 ml volumetric flask (5.6), add 100 µl of the 0,15 mM tryptamine solution (7.1) using a syringe (5.7) and make up to the volume with methanol (4.2.1). Mix carefully by inversion and sonicate (5.8) for 15 min*

7.2.3. *Centrifuge (5.9) at 27 000 g × g for 10 minutes and collect the supernatant in a glass vial (5.10)*

Note: Test sample solution should be stored at 4 °C until the HPLC analysis is performed.

7.3. **Preparation of the external standard solution**

7.3.1. *Weigh 55,4 mg PEDP (4.1) into a 50 ml volumetric flask (5.6) and add about 25 ml of chloroform (4.2.2) using a graduated cylinder (5.11). Heat the stoppered flask to 50 °C ± 1 °C and mix carefully till the PEDP dissolves. Cool the flask to 20 °C, make up to the volume with methanol (4.2.1) and mix by inversion*

7.3.2. *Pipette 1 ml (5.3) of this solution into a 100 ml volumetric flask (5.6) and make up to the volume with methanol (4.2.1). Pipette 1 ml (5.3) of this solution into a 10 ml volumetric flask (5.6), add 100 µl (5.7) of 0,15 mM tryptamine solution (7.1) and make up to the volume with methanol (4.2.1). Mix by inversion*

Note: Reference sample solution should be stored at 4 °C until the HPLC analysis is performed.

7.4. **Preparation of the derivatising reagent**

Weigh 25,0 ± 0,1 mg of OPA (4.3.4) into a 10 ml volumetric flask (5.6), add 0,5 ml (5.5) of methanol (4.2.1) and mix carefully to dissolve the OPA. Make up to the mark with boric acid solution (4.3.2) and add 20 µl of 2-mercaptoethanol (4.3.3) by syringe (5.7).

Note: The derivatising reagent should be stored at 4 °C in a brown glass vial and is stable for one week.

7.5. **Determination by HPLC**

7.5.1. *Elution solvents (4.4)*

Solvent A: Solution of 0,3 mM sodium dihydrogen phosphate and 3 mM sodium acetate solution (adjusted to pH 6,5 ± 0,1 with acetic acid): methanol: tetrahydrofuran = 558:440:2 (v/v/v)

Solvent B: methanol

7.5.2. *Suggested eluting gradient:*

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)
Initial	40	60	0
0,1	40	60	0,1
5,0	40	60	0,1
6,0	40	60	1,0
6,5	40	60	1,0
9,0	36	64	1,0
10,0	20	80	1,0
11,5	16	84	1,0
12,0	16	84	1,0
16,0	10	90	1,0
19,0	0	100	1,0
20,0	0	100	1,0
21,0	40	60	1,0
29,0	40	60	1,0
30,0	40	60	0

Note: The eluting gradient may require slight modification in order to achieve the resolution shown in figure 1.

Column temperature: 30 °C.

7.5.3. *Injection volume: 50 µl derivatising reagent and 50 µl sample solution*

7.5.4. *Column equilibration*

Starting up the system on a daily basis, flush the column with 100 % solvent B for 15 minutes, then set at A:B = 40:60 and equilibrate at 1 ml/min for 15 minutes. Perform a blank run by injecting methanol (4.2.1).

Note: Before long-term storage flush the column with methanol: chloroform = 80:20 (v/v) for 30 minutes.

7.5.5. *Determine the PS + PE content in the test sample*

7.5.6. *Perform the sequence of the chromatographic analyses keeping constant the run-to-run time in order to obtain constant retention times. Inject the external standard solution (7.3) every 5-10 test sample solutions in order to calculate the response factor*

Note: The column shall be cleaned by flushing with 100 % solvent B (7.5.1) for at least 30 minutes every 20-25 runs.

7.6. **Integration mode**

7.6.1. *PEDP peak*

PEDP is eluted as a single peak. Determine the peak area by valley-to- valley integration.

7.6.2. *Tryptamine peak*

Tryptamine is eluted as a single peak (Figure 1). Determine the peak area by valley-to-valley integration.

7.6.3. *PS and PE peaks groups*

Under the described conditions (Figure 1), PS elutes as two main partially unresolved peaks preceded by a minor peak. PE elutes as three main partially unresolved peaks. Determine the whole area of each peak cluster setting the baseline as reported in Figure 1.

8. CALCULATION AND EXPRESSION OF RESULTS

PS and PE content in the test sample shall be calculated as follows:

$$C = 55{,}36 \times ((A_2)/(A_1)) \times ((T_1)/(T_2))$$

where:

C = PS or PE content (mg/100 g powder) in the test sample

A_1 = PEDP peak area of the standard sample solution (7.3)

A_2 = PS or PE peak area of the test sample solution (7.2)

T_1 = Tryptamine peak area of the standard sample solution (7.3)

T_2 = Tryptamine peak area of the test sample solution (7.2).

9. ACCURACY OF THE METHOD

Note: The values for repeatability were calculated according to the IDF International Standard (*).

9.1. **Repeatability**

The relative standard deviation of the repeatability, which expresses the variability of independent analytical results obtained by the same operator using the same apparatus under the same conditions on the same test sample and in a short interval of time, should not exceed 2 % relative. If two determinations are obtained under these conditions, the relative difference between the two results should not be greater than 6 % of the arithmetic mean of the results.

9.2. **Reproducibility**

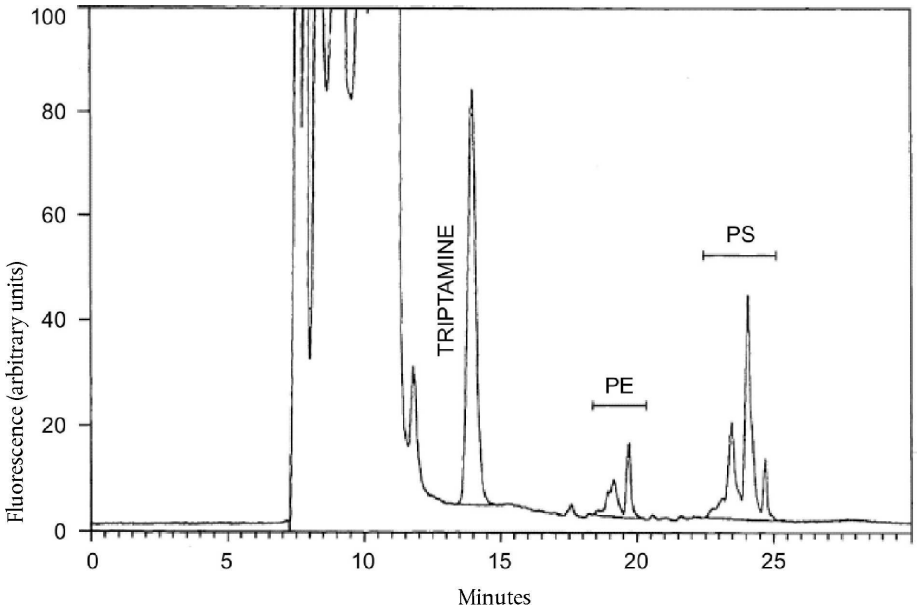
If two determinations are obtained by operators in different laboratories using different apparatus under different conditions for the analysis on the same test sample, the relative difference between the two results should not be greater than 11 % of the arithmetic mean of the results.

10. REFERENCES

10.1. *Resmini P., Pellegrino L., Hogenboom J.A., Sadini V., Rampilli M., 'Detection of buttermilk solids in skimmilk powder by HPLC quantification of aminophospholipids'. Sci. Tecn. Latt.-Cas., 39,395 (1988).*

Figure 1

HPLC pattern of OPA-derivatives of phosphatidylserine (PS) and phosphatidylethanolamine (PE) in methanol extract of reconstituted skim-milk powder. Integration mode for the peaks of PS, PE and tryptamine (internal standard) is reported



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**

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.

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 ≤ 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: $T_1 = S_{III}[E] - [(0,376\ P\% - 10,7) + (S_{III}[0] - 0,9)]$ $T_2 = S_{III}[E] - [(0,0123\ S_{II}[E] + 0,93) + (S_{III}[0] - 0,9)]$

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

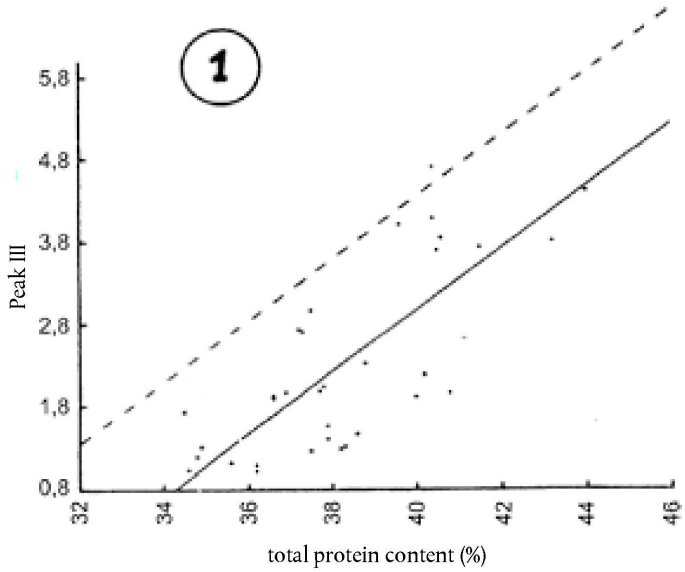
If T_1 and T_2 exceed zero, rennet whey is present.

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

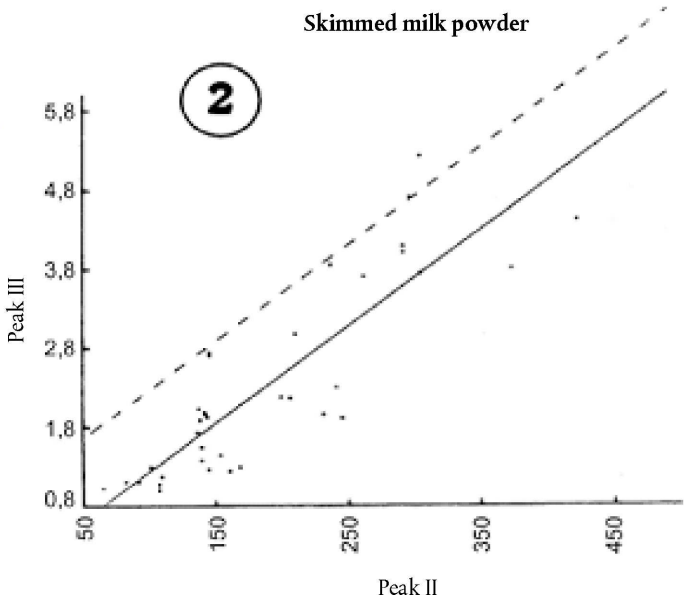
where:

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

Skimmed milk powder



Skimmed milk powder



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, 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**

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:*

$$CMP_A \text{ peak: } R = W/H$$

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*

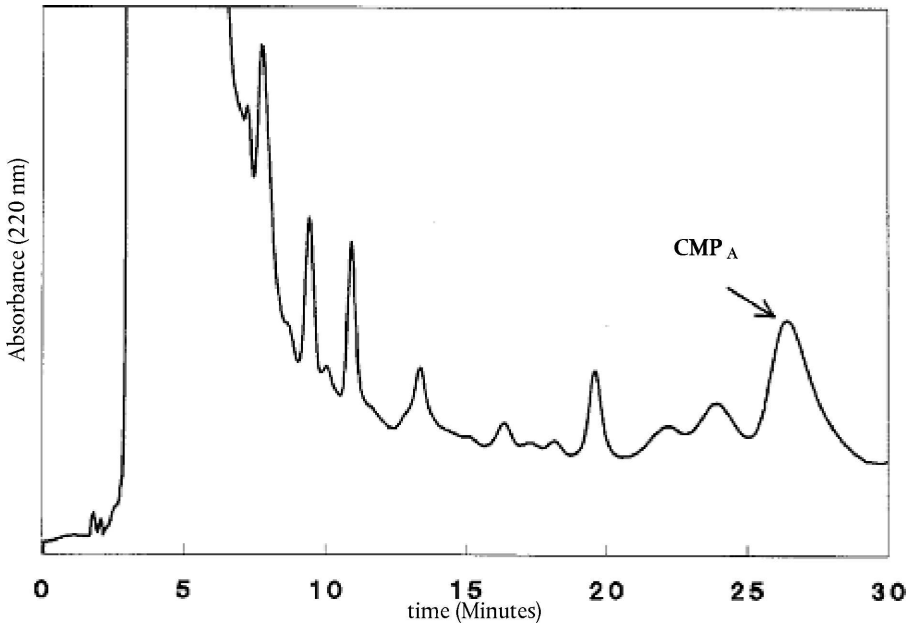
From 0 to 16 % of rennet whey a linear relationship should be obtained with a coefficient of correlation > 0,99.

9.4. **Interpretation**

The 1 % limit includes the uncertainty due to reproducibility.

Figure 1

Ni—4.6 standard



(*) International IDF Standard 135B/1991. Milk and milk products. Precision characteristics of analytical methods. Outline of collaborative study procedure.'

(3) the following Annexes are added:

'*ANNEX VI*

Methods of analysis of butter under private storage

Parameter	Method
Fat (1)	ISO 17189 or ISO 3727 part 3
Water	ISO 3727 part 1
Non Fat Solids (excluding salt)	ISO 3727 part 2
Salt	ISO 15648

(1) The method to be applied shall be approved by the paying agency.

ANNEX VII

Methods of analysis of skimmed milk powder under private storage

Parameter	Method
Fat	ISO 1736
Protein	ISO 8968 part 1
Water	ISO 5537

ANNEX VIII

Methods of analysis of cheeses under private storage

1. The method of analysis laid down in the Appendix shall be used to ensure that cheese made exclusively from ewe's milk, goat's milk or buffalo milk or from a mixture of ewe's milk, goat's milk and buffalo milk does not contain cow's milk casein.

   Cow's milk casein is considered to be present if the cow's milk casein content of the analysed sample is equal to or higher than the content of the reference sample containing 1 % cow's milk as laid down in the Appendix.

2. Methods for detecting cow's milk casein in cheeses referred to in paragraph 1 may be used provided that:

   (a) the detection limit is maximum 0,5 % and

   (b) there are no false-positive results and

   (c) cow's milk casein is detectable with the required sensitivity even after long ripening periods, as may occur in usual commercial conditions.

   If any of the above mentioned requirements is not met, the methods laid down in the Appendix shall be used.

Appendix

METHOD FOR THE DETECTION OF COW'S MILK AND CASEINATE IN CHEESES FROM EWE'S MILK, GOAT'S MILK OR BUFFALO MILK OR MIXTURES OF EWE'S MILK, GOAT'S MILK AND BUFFALO MILK

1. SCOPE

Detection of cow's milk and caseinate in cheeses made from ewe's milk, goat's milk, buffalo milk or mixtures of ewe's, goat's and buffalo milk by isoelectric focusing of γ-caseins after plasminolysis.

2. FIELD OF APPLICATION

The method is suitable for sensitive and specific detection of native and heat-treated cow's milk and caseinate in fresh and ripened cheeses made from ewe's milk, goat's milk, buffalo milk or mixtures of ewe's, goat's and buffalo milk. It is not suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates.

3. PRINCIPLE OF THE METHOD

3.1. **Isolation of caseins from cheese and the reference standards**

3.2. **Dissolving of the isolated caseins and submitting to plasmin (EC.3.4.21.7) cleavage**

3.3. **Isoelectric focusing of plasmin-treated caseins in the presence of urea and staining of proteins**

3.4. **Evaluation of stained γ_3 and γ_2 -casein patterns (evidence of cow's milk) by comparison of the pattern obtained from the sample with those obtained in the same gel from the reference standards containing 0 % and 1 % cow's milk.**

4. REAGENTS

Unless otherwise indicated, analytical grade chemicals shall be used. Water shall be double-distilled or of equivalent purity.

Note: The following details apply to laboratory prepared polyacrylamide gels containing urea, of dimensions 265 × 125 × 0,25 mm. Where other sizes and types of gel are used, the separation conditions may have to be adjusted.

Isoelectric focusing

4.1. **Reagents for production of the urea containing polyacrylamide gels**

4.1.1. *Stock gel solution*

Dissolve:

4,85 g acrylamide

0,15 g N, N′-methylene-bis-acrylamide (BIS)

48,05 g urea

15,00 g glycerol (87 % w/w),

in water and make up to 100 ml and store in a brown glass bottle in the refrigerator.

Note: A commercially available pre-blended acrylamide/BIS solution may be used in preference to the quoted fixed weights of the neurotoxic acrylamides. Where such a solution contains 30 % w/v acrylamide and 0,8 % w/v BIS, a volume of 16,2 ml shall be used for the formulation instead of the fixed weights. The shelf life of the stock solution is a maximum of 10 days; if its conductivity is more than 5 μS, de-ionize by stirring with 2 g Amberlite MB-3 for 30 minutes, then filter through a 0,45 μm membrane.

4.1.2. *Gel solution*

Prepare a gel solution by mixing additives and ampholytes (*) with the stock gel solution (see 4.1.1).

9,0 ml stock solution

24 mg β-alanine

500 µl ampholyte pH 3,5-9,5

250 µl ampholyte pH 5-7

250 µl ampholyte pH 6-8

Mix the gel solution and de-gas for two to three minutes in an ultrasonic bath or in vacuum.

Note: Prepare the gel solution immediately prior to pouring it (see 6.2).

4.1.3. *Catalyst solutions*

4.1.3.1. N, N, N′ N′ — tetramethylethylenediamine (Temed)

4.1.3.2. 40 % w/v ammonium persulphate (PER):

Dissolve 800 mg PER in water and make up to 2 ml.

Note: Always use freshly prepared PER solution.

4.2. **Contact fluid**

Kerosene or liquid paraffin

4.3. **Anode solution**

Dissolve 5,77 g phosphoric acid (85 % w/w) in water and dilute to 100 ml.

4.4. **Cathode solution**

Dissolve 2,00 g sodium hydroxide in water and dilute to 100 ml with water.

Sample preparation

4.5. **Reagents for protein isolation**

4.5.1. *Dilute acetic acid (25,0 ml of glacial acetic acid made up to 100 ml with water)*

4.5.2. *Dichloromethane*

4.5.3. *Acetone*

4.6. **Protein dissolving buffer**

Dissolve

5,75 g glycerol (87 % w/w)

24,03 g urea

250 mg dithiothreitol,

in water and make up to 50 ml

Note: Store in a refrigerator, maximum shelf-life one week.

4.7. **Reagents for plasmin cleavage of caseins**

4.7.1. *Ammonium carbonate buffer*

Titrate a 0,2 mol/l ammonium hydrogencarbonate solution (1,58 g/100 ml water) containing 0,05 mol/l ethylenediaminetetraacetic acid (EDTA, 1,46 g/100 ml with a 0,2 mol/l ammonium carbonate solution (1,92 g/100 ml water) containing 0,05 mol/l EDTA to pH 8.

4.7.2. *Bovine plasmin (EC. 3.4.21.7), activity at least 5 U/ml*

4.7.3. *ε-Aminocaproic acid solution for enzyme inhibition*

Dissolve 2,624 g ε-aminocaproic acid (6 amino-n-hexanoic acid) in 100 ml of 40 % (v/v) ethanol.

4.8. **Standards**

4.8.1. *Certified reference standards of a mixture of renneted ewe's and goat's skimmed milk containing 0 % and 1 % of cow's milk are available from the Commission's Institute for Reference Materials and Measurements, B-2440 Geel, Belgium*

4.8.2. *Preparation of laboratory interim-standards of buffalo's renneted milk containing 0 % and 1 % of cow's milk*

Skimmed milk is prepared by centrifuging of either buffalo or bovine raw bulk milk at 37 °C at 2 500 g for 20 minutes. After cooling the tube and contents rapidly to 6 to 8 °C, the upper fat layer is removed completely. For the preparation of the 1 % standard add 5,00 ml of bovine skimmed milk to a 495 ml of buffalo's skimmed milk in a 1 l beaker, adjust the pH to 6,4 by the addition of dilute lactic acid (10 % w/v). Adjust the temperature to 35 °C and add 100 µl of calf rennet (rennet activity 1: 10 000, c. 3 000 U/ml), stir for 1 minute and then leave the beaker covered with an aluminium foil at 35 °C for one hour to allow formation of the curd. After the curd has formed, the whole renneted milk is freeze-dried without prior homogenization or draining of the whey. After freeze-drying it is finely ground to produce a homogeneous powder. For the preparation of the 0 % standard, carry out the same procedure using genuine buffalo skimmed milk. The standards shall be stored at − 20 °C.

Note: It is advisable to check the purity of the buffalo milk by isoelectric focusing of the plasmin-treated caseins before preparation of the standards.

Reagents for protein staining

4.9. **Fixative**

Dissolve 150 g trichloroacetic acid in water and make up to 1 000 ml.

4.10. **Destaining solution**

Dilute 500 ml methanol and 200 ml glacial acetic acid to 2 000 ml with distilled water.

Note: Prepare the destaining solution fresh every day; it can be prepared by mixing equal volumes of stock solutions of 50 % (v/v) methanol and 20 % (v/v) glacial acetic acid.

4.11. **Staining solutions**

4.11.1. *Staining solution (stock solution 1)*

Dissolve 3,0 g Coomassie Brilliant Blue G-250 (C.I. 42655) in 1 000 ml 90 % (v/v) methanol using a magnetic stirrer (approximately 45 minutes), filter through two medium-speed folded filters.

4.11.2. *Staining solution (stock solution 2)*

Dissolve 5,0 g copper sulphate pentahydrate in 1 000 ml 20 % (v/v) acetic acid.

4.11.3. *Staining solution (working solution)*

Mix together 125 ml of each of the stock solutions (4.11.1, 4.11.2) immediately prior to staining.

Note: The staining solution should be prepared on the day that it is used.

5. EQUIPMENT

5.1. **Glass plates (265 × 125 × 4 mm); rubber roller (width 15 cm); levelling table**

5.2. **Gel carrier sheet (265 × 125 mm)**

5.3. **Covering sheet (280 × 125 mm). Stick on strip of adhesive tape (280 × 6 × 0,25 mm) to each long edge (see Figure 1)**

5.4. **Electrofocusing chamber with cooling plate (e.g. 265 × 125 mm) and suitable power supply (≥ 2,5 kV) or automatic electrophoresis device**

5.5. **Circulation cryostat, thermostatically controlled at 12 ± 0,5 °C**

5.6. **Centrifuge, adjustable to 3 000 g**

5.7. **Electrode strips (≥ 265 mm long)**

5.8. **Plastic dropping bottles for the anode and cathode solutions**

5.9. **Sample applicators (10 × 5 mm, viscose or low protein-adsorption filter paper)**

5.10. **Stainless steel or glass staining and destaining dishes (e.g. 280 × 150 mm instrument trays)**

5.12. **Adjustable rod homogenizer (10 mm shaft diameter), rpm range 8 000 to 20 000**

5.13. **Magnetic stirrer**

5.14. **Ultrasonic bath**

5.15. **Film welder**

5.16. **25 µl micropipettes**

5.17. **Vacuum concentrator or freeze-dryer**

5.18. **Thermostatically controlled water bath adjustable to 35 and 40 ± 1 °C with shaker**

5.19. **Densitometer equipment reading at λ = 634 nm**

6. PROCEDURE

6.1. **Sample preparation**

6.1.1. *Isolation of caseins*

Weigh the amount equivalent to 5 g dry mass of cheese or the reference standards into a 100 ml centrifuge tube, add 60 ml distilled water and homogenize with a rod homogenizer (8 000 to 10 000 rpm). Adjust to pH 4,6 with dil. acetic acid (4.5.1) and centrifuge (5 minutes, 3 000 g). Decant the fat and whey, homogenize the residue at 20 000 rpm in 40 ml distilled water adjusted to pH 4,5 with dil. acetic acid (4.5.1), add 20 ml dichloromethane (4.5.2), homogenize again and centrifuge (5 minutes, 3 000 g). Remove the casein layer that lies between the aqueous and organic phases (see Figure 2) with a spatula and decant off both phases. Rehomogenise the casein in 40 ml distilled water (see above) and 20 ml dichloromethane (4.5.2) and centrifuge. Repeat this procedure until both extraction phases are colourless (two to three times). Homogenize the protein residue with 50 ml acetone (4.5.3) and filter through a medium-speed folded filter paper. Wash the residue on the filter with two separate 25 ml portions of acetone each time and allow to dry in the air or a stream of nitrogen, then pulverize finely in a mortar.

Note: Dry casein isolates should be kept at −20 °C.

6.1.2. *Plasmin cleavage of β-caseins to intensify γ-caseins*

Disperse 25 mg of isolated caseins (6.1.1) in 0,5 ml ammonium carbonate buffer (4.7.1) and homogenize for 20 minutes by e.g. using ultrasonic treatment. Heat to 40 °C and add 10 µl plasmin (4.7.2), mix and incubate for one hour at 40 °C with continuous shaking. To inhibit the enzyme add 20 µl ε-aminoproic acid solution (4.7.3), then add 200 mg of solid urea and 2 mg of dithiothreitol.

Note: To obtain more symmetry in the focused casein bands it is advisable to freeze-dry the solution after adding the ε-aminocaproic acid and then dissolving the residues in 0,5 ml protein dissolving buffer (4.6).

6.2. **Preparation of the urea containing polyacrylamide gels**

With the aid of a few drops of water roll the gel carrier sheet (5.2) onto a glass plate (5.1), removing any extraneous water with paper towel or tissue. Roll the cover sheet (5.3) with spacers (0,25 mm) onto another glass plate in the same way. Lay the plate horizontally on a levelling table.

Add 10 µl Temed (4.1.3.1) to the prepared and de-aerated gel solution (4.1.2), stir and add 10 µl PER-solution (4.1.3.2), mix thoroughly and immediately pour out evenly onto the centre of the cover sheet. Place one edge of the gel carrier plate (sheet side down) on the cover sheet plate and lower it slowly so that a gel film forms between the sheets and spreads out regularly and free of bubbles (Figure 3). Carefully lower the gel carrier plate completely using a thin spatula and place three more glass plates on top of it to act as weights. After polymerization is complete (about 60 minutes) remove the gel polymerized onto the gel carrier sheet along with the cover sheet by tipping the glass plates. Clean the reverse of the carrier sheet carefully to remove gel residues and urea. Weld the gel sandwich into a film tube and store in a refrigerator (maximum six weeks).

Note: The cover sheet with the spacers can be re-used. The polyacrylamide gel can be cut to smaller sizes, recommended when there are few samples or if an automatic electrophoresis device is used (two gels, size 4,5 × 5 cm).

6.3. **lsoelectric focusing**

Set the cooling thermostat to 12 °C. Wipe off the reverse of the gel carrier sheet with kerosene, then drip a few drops of kerosene (4.2) onto the centre of the cooling block. Then roll the gel sandwich, carrier side down, onto it, taking care to avoid bubbles. Wipe off any excess kerosene and remove the cover sheet. Soak the electrode strips with the electrode solutions (4.3, 4.4), cut to gel length and place in the positions provided (distance of electrodes 9,5 cm).

Conditions for isoelectric focusing:

6.3.1. *Gel size 265 × 125 × 0,25 mm*

Step	Time (min.)	Voltage (V)	Current (mA)	Power (W)	Volt-hours (Vh)
1. Pre-focusing	30	maximum 2 500	maximum 15	constant 4	c. 300
2. Sample focusing [1]	60	maximum 2 500	maximum 15	constant 4	c. 1 000
3. Final focusing	60	maximum 2 500	maximum 5	maximum 20	c. 3 000
	40	maximum 2 500	maximum 6	maximum 20	c. 3 000
	30	maximum 2 500	maximum 7	maximum 25	c. 3 000

[1] Sample application: After pre-focusing (step 1), pipette 18 µl of the sample and standard solutions onto the sample applicators (10 × 5 mm), place them on the gel at 1 mm intervals from each other and 5 mm longitudinally from the anode and press lightly. Carry out focusing using the above conditions, carefully removing the sample applicators after the 60 minutes of sample focusing.

Note: If thickness or width of the gels are changed, the values for current and power have to be suitably adjusted (e.g. double the values for electric current and power if a 265 × 125 × 0,5 mm gel is used).

6.3.2. *Example of a voltage programme for an automatic electrophoresis device (2 gels of 5,0 × 4,5 cm), electrodes without strips applied directly to the gel*

Step	Voltage	Current	Power	Temp.	Volt-hours
1. Pre-focusing	1 000 V	10,0 mA	3,5 W	8 °C	85 Vh
2. Sample focusing	250 V	5,0 mA	2,5 W	8 °C	30 Vh
3. Focusing	1 200 V	10,0 mA	3,5 W	8 °C	80 Vh
4. Focusing	1 500 V	5,0 mA	7,0 W	8 °C	570 Vh

Place sample applicator in step 2 at 0 Vh.

Remove sample applicator in step 2 at 30 Vh.

6.4. **Protein staining**

6.4.1. *Protein fixation*

Remove the electrode strips immediately after turning off the power and put the gel immediately into a staining/destaining dish filled with 200 ml fixative (4.9); leave for 15 minutes, shaking continuously.

6.4.2. *Washing and staining the gel plate*

Thoroughly drain off the fixative and wash the gel plate twice for 30 seconds each time with 100 ml destaining solution (4.10). Pour off the destaining solution and fill the dish with 250 ml staining solution (4.11.3); allow to stain for 45 minutes with gentle shaking.

6.4.3. *Destaining the gel plate*

Pour off the staining solution, wash the gel plate twice using a 100 ml destaining solution (4.10) each time, then shake with 200 ml destaining solution for 15 minutes and repeat the destaining step at least two or three times until the background is clear and uncoloured. Then rinse the gel plate with distilled water (2 × 2 minutes) and dry in the air (2 to 3 hours) or with a hairdryer (10 to 15 minutes).

Note 1: Carry out fixing, washing, staining and destaining at 20 °C. Do not use elevated temperatures.

Note 2: If more sensitive silver staining (e.g. Silver Staining Kit, Protein, Pharmacia Biotech, Code No 17-1150-01) is preferred, plasmin-treated casein samples have to be diluted to 5 mg/ml.

7. EVALUATION

Evaluation is performed by comparing the protein patterns of the unknown sample with reference standards on the same gel. Detection of cow's milk in cheeses from ewe's milk, goat's milk and buffalo milk and mixtures of ewe's, goat's and buffalo milk is done via the γ_3- and γ_2-caseins, whose isoelectric points range between pH 6,5 and pH 7,5 (Figures 4 a, b, Figure 5). The detection limit is less than 0,5 %.

7.1. **Visual estimation**

For visual evaluation of the amount of bovine milk it is advisable to adjust the concentrations of samples and standards to obtain the same level of intensity of the ovine, caprine and/or buffalo γ_2- and γ_3-caseins (see 'γ_2 E, G,B' and 'γ_3 E,G,B' in Figures 4 a, b and Figure 5). After which the amount of bovine milk (less than, equal to or greater than 1 %) in the unknown sample can be judged directly by comparing the intensity of the bovine γ_3- and γ_2-caseins (see 'γ_3 C' and 'γ_2 C' in Figures 4 a, b and Figure 5) to those of the 0 % and 1 % reference standards (ewe, goat) or, laboratory interim-standards (buffalo).

7.2. **Densitometric estimation**

If available, apply densitometry (5.19) for the determination of the peak area ratio of bovine to ovine, caprine and/or buffalo γ_2- and γ_3-caseins (see Figure 5). Compare this value to γ_2- and γ_3-casein peak area ratio of the 1 % reference standard (ewe, goat) or laboratory interim-standard (buffalo) analysed on the same gel.

Note: The method is operating satisfactorily, if there is a clear positive signal for both bovine γ_2- and γ_3-caseins in the 1 % reference standard but not in the 0 % reference standard. If not, optimize the procedure following the details of the method precisely.

A sample is judged as being positive, if both bovine γ_2- and γ_3-caseins or the corresponding peak area ratios are equal to or greater than the level of the 1 % reference standard.

8. REFERENCES

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Krause I., Berner I, Klostermeyer H.: Sensitive detection of cow milk in ewe and goat milk and cheese by carrier ampholyte — and carrier ampholyte/immobilized pH gradient — isoelectric focusing of γ-caseins using plasmin as signal amplifier. in: Electrophoresis-Forum 89 (B. J. Radola, ed.) pp 389-393, Bode-Verlag, München (1989).

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Figure 1

Schematic drawing of the covering sheet

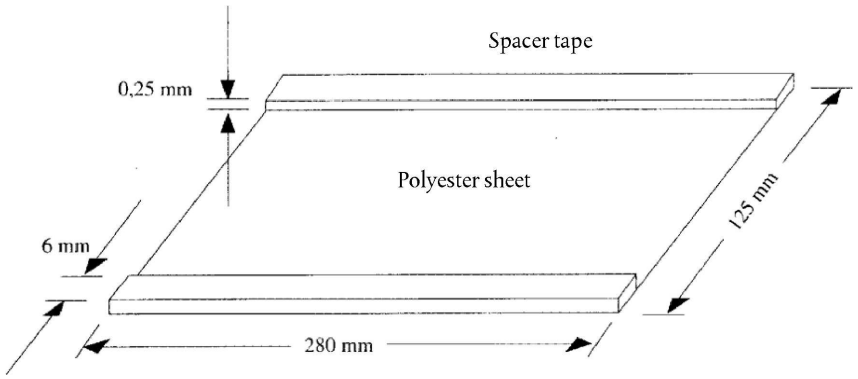


Figure 2

Casein layer floating between aqueous and organic phases after centrifugation

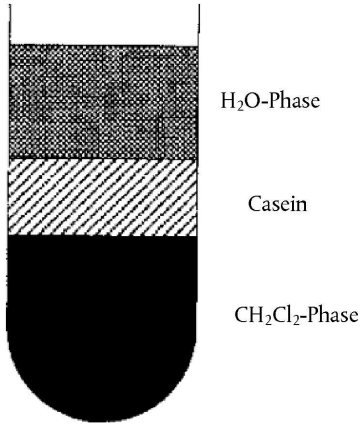
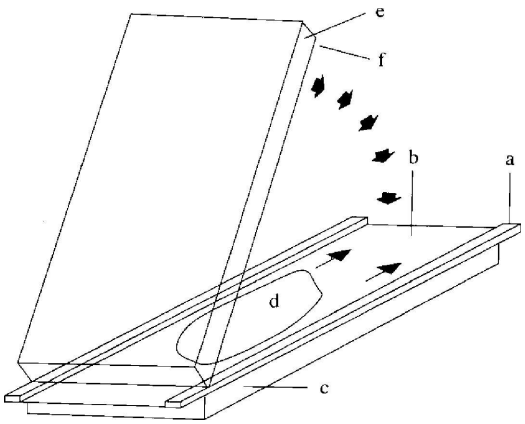


Figure 3

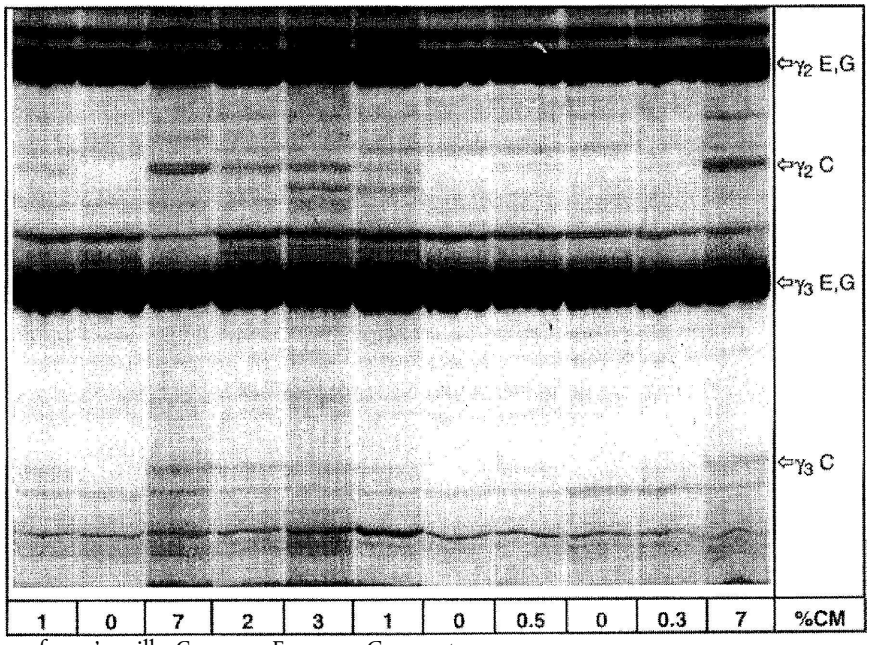
Flapping technique for casting of ultrathin polyacrylamide gels



a = spacer tape (0,25 mm); b = covering sheet (5.3); c, e = glass plates (5.1); d = gel solution (4.1.2); f = gel carrier sheet (5.2)

Figure 4a

Isoelectric focusing of plasmin-treated caseins from ewe's and goat's milk cheese containing different amounts of cow's milk.

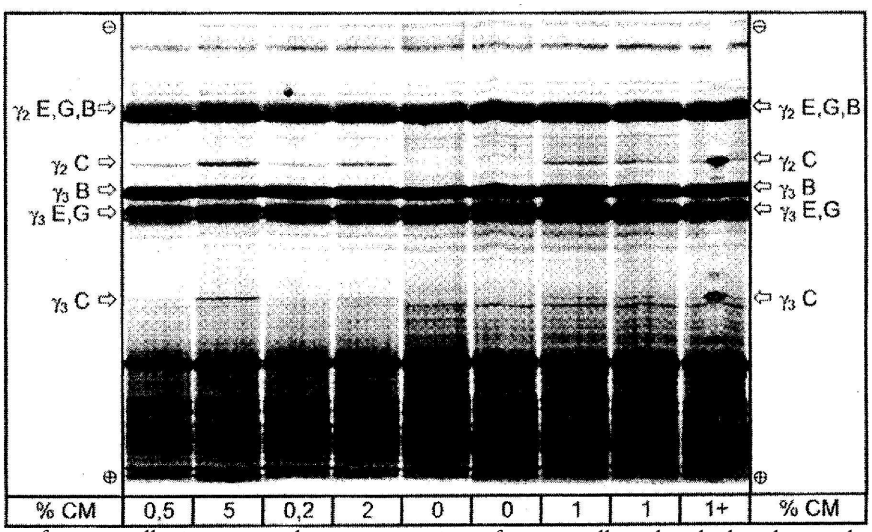


% CM = percentage of cow's milk, C = cow, E = ewe, G = goat

Upper half of the IEF gel is shown.

Figure 4b

Isoelectric focusing of plasmin treated caseins from cheese made from mixtures of ewe's, goat's and buffalo milk containing different amounts of cow's milk.

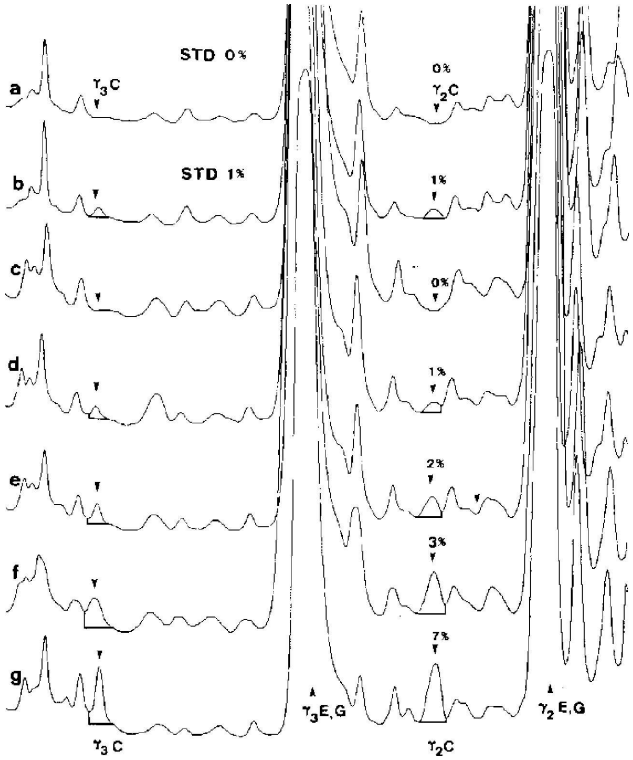


% CM = percentage of cow's milk; 1 + = sample containing 1 % of cow's milk and spiked with pure bovine casein at the middle of the track. C = cow, E = ewe, G = goat, B = buffalo.

Total separation distance of the IEF gel is shown.

Figure 5

Superposition of densitograms of standards (STD) and cheese samples made from a mixture of ewe's and goat's milk after isoelectric focusing.



a,b = standards containing 0 and 1 % of cow's milk; c-g = cheese samples containing 0, 1, 2, 3 and 7 % of cow's milk. C = cow, E = ewe, G = goat.

Upper half of the IEF gel was scanned at λ = 634 nm.

ANNEX IX

Evaluation of the analyses

1. **Quality assurance**

Analyses shall be performed by laboratories designated in accordance with Article 12 of Regulation (EC) No 882/2004 (**) or designated by the competent authorities of the Member State.

2. **Sampling and disputes over the results of analysis**

1. Sampling shall be carried out in accordance with the relevant regulation for the product under consideration. If no sampling provisions are expressly provided for, then the provisions laid down in ISO 707, Milk and milk products – Guidance on sampling, shall be used.

2. Laboratory reports of the results of the analysis shall contain sufficient information to allow an evaluation of the results to be carried out in accordance with the Appendix.

3. Duplicate samples shall be taken for analyses required under Union rules.

4. If a dispute arises over the results, the paying agency shall have the necessary analysis on the product in question carried out again, and the cost shall be met by the losing party.

The above mentioned analysis shall be carried out provided that sealed duplicate samples of the product are available and have been stored appropriately with the competent authority. The manufacturer shall send a request to the paying agency to conduct the analysis within 7 working days following the notification of the results of the first analysis. The analysis shall be carried out by the paying agency within 21 working days following receipt of the request.

5. The appeal result shall be the definitive one.

6. If the manufacturer can prove, within five working days of sampling, that the sampling procedure was not carried out correctly, sampling shall be repeated where possible. If sampling cannot be repeated, the consignment shall be accepted.

Appendix

Evaluation of compliance of a consignment with the legal limit

1. **Principle**

Where public intervention and private storage legislation lay down detailed sampling procedures then those procedures shall be followed. In all other cases a sample of at least 3 sample units taken randomly from the consignment submitted to control shall be used. A composite sample may be prepared. The result obtained shall be compared with the legal limits by calculation of a 95 % confidence interval as 2 x standard deviation, where the relevant standard deviation depends on whether (1) the method is validated through international collaboration with values for σ_r and σ_R or (2) in the case of in-house validation, an internal reproducibility has been calculated. This confidence interval will then equate to the measurement uncertainty of the result.

2. **The method is validated through international collaboration**

In this case, the repeatability standard deviation σ_r and the reproducibility standard deviation σ_R have been established and the laboratory can demonstrate compliance with the performance characteristics of the validated method.

Calculate the arithmetic mean $\bar{x}$ of the n repeated measurements.

Calculate the expanded uncertainty ($k = 2$) of $\bar{x}$ as

$$U = 2\sqrt{\sigma_R^2 - \frac{n-1}{n}\sigma_r^2}$$

If the final result x of measurement is calculated using a formula of the form $x = y_1 + y_2$, $x = y_1 - y_2$, $x = y_1 \cdot y_2$ or $x = y_1/y_2$ the usual procedures for combining standard deviations in such cases shall be followed.

The consignment is judged to be not in compliance with the upper legal limit UL if

$\bar{x} - U > UL$;

otherwise it is judged to be in compliance with UL.

The consignment is judged to be not in compliance with the lower legal limit LL if

$\bar{x} + U < LL$;

otherwise it is judged to be in compliance with LL.

3. **In-house validation with calculation of internal reproducibility standard deviation**

In cases where methods not specified in this Regulation are used and precision measures have not been established, an in-house validation shall be carried out. Internal repeatability standard deviation σ_{ir} and the internal reproducibility standard deviation σ_{iR} shall be used instead of σ_r and σ_R, resp., in the formulae for the computation of the expanded uncertainty U.

The rules to be followed to determine compliance with the legal limit are as set out under point 1. However, if the consignment is judged to be non-compliant with the legal limit, the measurements shall be repeated with the method specified in this Regulation and the result evaluated in accordance to point 1.

(*) The produce Ampholine® pH 3,5-9,5 (Pharmacia) and Resolyte® pH 5-7 and pH 6-8 (BDH, Merck) have proved particularly suitable for obtaining the required separation of γ-caseins.

(**) Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules (OJ L 165, 30.4.2004, p. 1).'