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# Milk-based infant formula powders — Quantification of whey protein content by sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE)

*Formules infantiles en poudre à base de lait — Quantification de la teneur en protéine de lactosérum par électrophorèse capillaire sur gel contenant du dodécylsulfate de sodium (SDS-CGE)*



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# Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC INTERNATIONAL. It is being published jointly by ISO and IDF, and separately by AOAC INTERNATIONAL. The method described in this document is equivalent to the AOAC Official Method AOAC 2016.15: *Quantification of Whey Protein Content in Milk-Based Infant Formula Powders by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis (SDS-CGE), First Action 2016, Final Action 2018*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.



 

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The work was carried out by the IDF/ISO Action Team (C31) of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader Mr E. Konings (CH).

# Milk-based infant formula powders — Quantification of whey protein content by sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE)

## 1 Scope

This document specifies a method for the determination of the whey to casein protein ratio, ranging from 20:80 to 80:20 in cow milk-based infant formula powders.

This method does not apply to the analysis of infant formulas containing hydrolysed protein or proteins from other sources (e.g. plants or milk from other mammals).

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

**3.1**
**infant formula**
breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

## 4 Principle

In sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE), proteins in infant formula samples are denatured by the anionic surfactant SDS and reduced by β-mercaptoethanol. The SDS-bonded, electrically charged proteins migrate in an electrical field filled with a separation gel and are detected by UV at 220 nm. Caseins and whey proteins are separated as two distinct, non-overlapping groups of peaks where the ratio can be established based on integrated areas without the need for a calibration curve. In the calculation of whey protein content, 1,4 was used as the mass-to-area correction factor for whey proteins versus caseins.

## 5 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

Data from the multi-laboratory study was obtained with chemicals from the SDS-MW kit 390953 from Beckman Coulter/Sciex[1)]. The SDS-MW gel separation buffer recipe described below (5.1) was tested in a separate study and found to be equivalent to the commercial SDS-MW gel separation buffer included in the kit. One laboratory used home-made reagents (5.2 to 5.5). All laboratories used the 10 kDa internal protein standard (5.6) and the SDS-MW size standard (5.7) from the kit. Use of equivalent standards requires prior verification.

**5.1 SDS-MW gel separation buffer,** tris(hydroxymethyl)aminomethane (Tris), boric acid, SDS, EDTA, glycerol and dextran 2000[1)] .

Dissolve 0,726 6 g Tris base and 0,371 0 g boric acid in 9 ml water. Add 20 mg SDS, 1 g dextran 2000, 14,6 mg EDTA, and 1 ml glycerol. Mix thoroughly. Leave overnight to allow complete dextran solubilization.

NOTE Dextran 2000 has an average molecular weight of 2 000 000 Da.

**5.2 SDS-MW sample buffer,** 100 mmol/l Tris, pH 9,0, 1 % SDS.

Dissolve 109,4 mg of Tris base, 15,2 mg of Tris hydrocholoride and 0,1 g of SDS in 10 ml water.

**5.3 Acidic wash solution**, hydrochloric acid, substance concentration $c$ = 0,1 mol/l.

**5.4 Basic wash solution**, sodium hydroxide, $c$ = 0,1 mol/l.

**5.5 β-mercaptoethanol.**

**5.6 10 kDa internal protein standard**, mass concentration $\rho$ = 5 mg/ml.

**5.7 SDS-MW size standard**, 10 to 225 kDa, $\rho$ = 16 mg/ml.

**5.8 Sample running pre-solution.**

Mix the SDS-MW sample buffer (5.2) with the 10 kDa internal protein standard (5.6) using an 84:1 ratio. Prepare a sufficient volume based on the total number of samples to be analysed in the sample set (90 µl of sample running pre-solution is needed per sample).

## 6 Apparatus

Usual laboratory glassware and equipment and, in particular, the following.

**6.1 Capillary electrophoresis instrument**, equipped with UV detector set at 220 nm and capable of maintaining the capillary and sample tray at 25 °C ± 2 °C.

Suitable software should be available for peak integration.

**6.2 Bare**, fused-silica capillaries, of 50 µm internal diameter × 30 cm, with effective length of 20 cm.

**6.3 Temperature controlled water bath or heating block**, capable of maintaining a temperature of 95 °C ± 5 °C.

**6.4 Micro centrifuge**, with adaptors for 1,5 ml centrifuge vials.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.



**6.5 Vortex.**

**6.6 Adjustable pipettes and tips**, volume of 20 µl, 100 µl and 1 000 µl.

**6.7 Safe-lock micro-centrifuge vials**, volume of 1,5 ml or 2,0 ml.

**6.8 Round-bottom centrifuge tubes**, volume of 10 ml.

**6.9 Analytical balance**, weighing to four decimal places.

## 7 Procedure

### 7.1 Sample preparation

**7.1.1** Weigh 500 mg ± 20 mg of infant formula powder into a 10 ml centrifuge tube. A skim milk powder (SMP) sample is used as a reference to align the whey and casein regions in the infant formula samples. Prepare the SMP using a sample mass of 135 mg ± 5 mg.

**7.1.2** Disperse the sample in 5 ml of water. Vortex each tube until the solution appears homogeneous. Each final solution contains between 10 mg/ml and 15 mg/ml of protein.

**7.1.3** Pipette 10 µl of each sample solution into separate microcentrifuge vials.

**7.1.4** Sequentially add 85 µl of the sample running pre-solution (5.8) and 5 µl of β-mercaptoethanol (5.5) to each micro-centrifuge vial. Mix well before heating the vials in a water bath or heating block at 95 °C ± 5 °C for 10 min. Cool down to room temperature. Centrifuge at room temperature for 1 min at about 5 000*g* (approximately 7 000 r/min in a table-top centrifuge).

**7.1.5** Vortex before transferring each sample into corresponding injection vials.

### 7.2 CGE analysis

**7.2.1** The separation and the quantification have proven to be satisfactory if the following experimental conditions are followed.

**7.2.2** Load the reagents according to the instructions of the capillary electrophoresis instrument manufacturer (see Figure A.1 for an example).

**7.2.3** Set up an optimized batch analysis separation method including a reagent blank (10 µl of water replacing the sample solution), a molecular weight size standard (5.7) and a SMP sample.

To prepare the reagent blank, replace the sample solution (see 7.1.3) by 10 µl water. Its function is to monitor any contamination in the buffer or reagents (there should be no peaks in the migration range of milk protein as defined from the 10 kDa internal standard protein). SMP works as a reference to verify the migration times for whey proteins and caseins.

**7.2.4** For each separation cycle (45 min), precondition the capillary first with the basic wash solution (5.4) for 3 min, followed by the acidic wash solution (5.3) for 1 min and water for 1 min. Fill the capillary with the SDS-MW gel separation buffer (5.1) for 10 min. Set the separation run time to 30 min.

**7.2.5** Introduce the samples electrokinetically by applying voltage at −5 kV for 20 s.

**7.2.6** Perform the electrophoresis at constant voltage with applied field strength of −497 V/cm and a capillary thermostatted to 25 °C using recirculating liquid coolant.

**7.2.7** The generated current should be approximately 27 μA.

**7.2.8** To avoid reagent depletion, programme the system to increment the vial position of all reagents every eight cycles.

## 7.3 Electropherogram processing

### 7.3.1 Electropherogram integration

Automatically integrate the electropherograms from the valley located at 0,5 min to 1 min before the 10 kDa internal protein standard peak (include any peak immediately adjacent to the 10 kDa internal protein standard) to the valley between the end of κ-casein peak and the peak of Ig H and BSA (no negative peaks should be generated by the integration). Then, perform a manual integration from the valley before the peak of Ig H and BSA until the end of the last peak in the electropherogram (integrate all peaks including those after the peak of Ig H and BSA if present). Examples of electropherograms including the automated and manual integration regions are given in Figures A.2 and A.3 for SMP and infant formulas, respectively. Examples of integration parameters are listed in Table A.1.

### 7.3.2 Identification of the casein region

In SMP, the casein integration area starts just before the β-casein peak (the highest peak of the electropherogram) and stops at the valley between the end of the κ-casein peak and the peak of Ig H and BSA. For infant formulas, identify the β-casein peak and the valley by comparison with the SMP electropherogram.

# 8 System suitability

## 8.1 General

Test the system suitability using the 10 kDa internal protein standard (5.6) and the SDS-MW size standard (5.7). Define the migration time of the 10 kDa internal protein standard for each combination of instrument, capillary and reagents beforehand. The acceptance criteria for the system suitability are as follows.

**8.2** The migration time of the 10 kDa internal protein standard should correspond to the value initially established ±5 %. The seven MW markers (10 kDa, 20 kDa, 35 kDa, 50 kDa, 100 kDa, 150 kDa and 225 kDa) should be completely separated (see Figure A.4).

EXAMPLE Using a Beckman Coulter/Sciex instrument, capillaries and reagents[2)], the migration time of the 10 kDa internal protein standard is 12,3 min ± 0,5 min and the seven MW markers are completely separated within 30 min (see Figure A.4).

**8.3** The acceptance criteria for the separation cycles are:

— the migration time of the 10 kDa internal protein standard should correspond to the value initially established ±5 %;

— the degree of baseline drop from the internal standard until the valley between the end of κ-casein and the peaks of Ig H and BSA should not exceed 25 % of the height of the internal protein standard in the sample.

2) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product.

# 9 Calculations and expression of results

## 9.1 Calculation of whey protein content

Separately sum peak areas in three regions, two at each end of the electropherogram (lower molecular weight and higher molecular weight whey proteins) and one in the middle. The middle region corresponds to casein proteins ($A_{cn}$), and the two others are summed together to obtain whey proteins ($A_w$). The area of the internal standard should not be included in the calculation of $A_w$. Refer to the blank run to identify potential degradation products of the 10 kDa internal protein standard. If present, they should not be included in the calculation of $A_w$.

Calculate the whey protein, $W_P$, content as a percentage using Formulae (1) and (2):

$$W_P = \frac{A_{w,c}}{A_{w,c} + A_{cn}} \times 100 \quad (1)$$

$$A_{w,c} = A_w \times 1,4 \quad (2)$$

where

$A_w$ is the total integrated areas of whey components;

$A_{w,c}$ is the corrected integrated area of whey components;

$A_{cn}$ is the integrated area of casein components;

1,4 is the correction factor to account for the difference between the mass-to-area ratio of whey and casein proteins.

NOTE The peaks in the electropherograms could shift backwards gradually from the beginning of the run to the end of the run. The internal standard peak is used to correct the migration times for all the peaks in the electropherogram.

## 9.2 Expression of results

Express the obtained results in per cent (grams of whey per 100 g of protein) to two decimal places, if needed for further calculations. In cases where the results are end results, express the results to one decimal place.

# 10 Precision

## 10.1 Interlaboratory tests

Interlaboratory tests were conducted in accordance with ISO 5725-1 and ISO 5725-2. The repeatability and reproducibility values for 14 cow milk-based infant formulas with a whey protein content of 40 % or more and one reference standard for infant/adult nutritional formula with a whey protein content below 40 % have been published[7]. The performance values for the SMP sample that had been systematically used as a quality control during the same study were subsequently extracted. The values from the SMP and the reference standard samples were used to evaluate method performance in infant formulas with a whey protein content below 40 %. The values derived from these trials might not be applicable to concentration ranges and matrices other than those given. In particular, this method is not applicable to infant formulas produced from milk originating from species other than cow, from plant proteins or from hydrolyzed proteins.

### 10.2 Infant formulas with a whey protein content of 40 % or more

#### 10.2.1 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 3,70 % whey content (2,8 times the highest $s_r$).

#### 10.2.2 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 6,22 % whey content (2,8 times the highest $s_R$).

### 10.3 Infant formulas with a whey protein content below 40 %

#### 10.3.1 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 4,20 % whey content (2,8 times the highest $s_r$).

#### 10.3.2 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 7,11 % whey content (2,8 times the highest $s_r$).

## 11 Test report

The test report shall specify:

a) all the information required for the complete identification of the sample;

b) the sampling method used, if known;

c) the method used, with reference to this document, i.e. ISO 23293 | IDF 247;

d) all operating details not specified in this document, or regarded as optional, together with details of any incident which might have influenced the result(s);

e) the test result(s) obtained and, if the repeatability or the recovery has been checked, the final quoted result obtained;

f) the date of the test.

# Annex A
(informative)

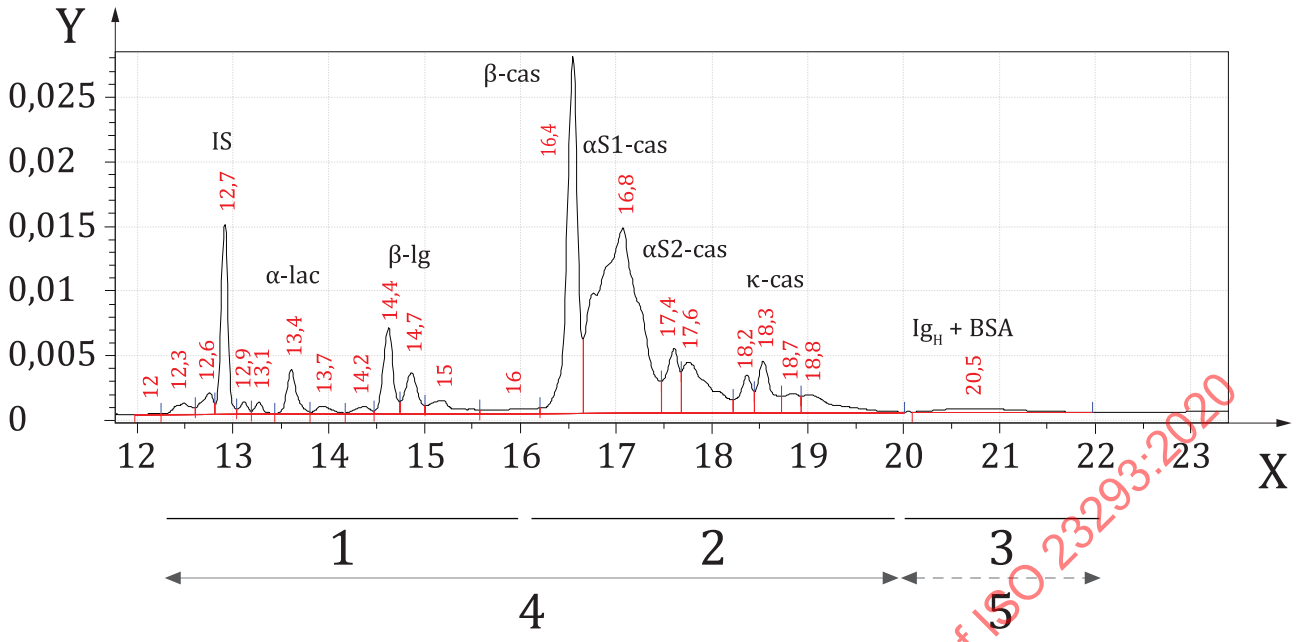
# Example of a CGE analysis

| Inlet buffer tray | | | | | | Outlet buffer tray | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $H_2O$ (cycles 17 to 24) 1,5 ml | $H_2O$ (cycles 17 to 24) 1,5 ml | | | | | $H_2O$ (cycles 17 to 24) 1,5 ml | $H_2O$ (cycles 17 to 24) 1,5 ml | | | | |
| $H_2O$ (cycles 9 to 16) 1,5 ml | $H_2O$ (cycles 9 to 16) 1,5 ml | | | | | $H_2O$ (cycles 9 to 16) 1,5 ml | $H_2O$ (cycles 9 to 16) 1,5 ml | | | | |
| $H_2O$ (cycles 1 to 8) 1,5 ml | $H_2O$ (cycles 1 to 8) 1,5 ml | | | | | $H_2O$ (cycles 1 to 8) 1,5 ml | $H_2O$ (cycles 1 to 8) 1,5 ml | | | | |
| $H_2O$ (cycles 17 to 24) 1,5 ml | Gel (cycles 17 to 24) 1,2 ml | Gel (cycles 17 to 24) 1,1 ml | NaOH (cycles 17 to 24) 1,5 ml | HCl (cycles 17 to 24) 1,5 ml | $H_2O$ (cycles 17 to 24) 1,5 ml | $H_2O$ (cycles 17 to 24) 1,5 ml | $H_2O$ waste (17 to 24) 1,0 ml | Gel (cycles 17 to 24) 1,1 ml | $H_2O$ waste (17 to 24) 1,0 ml | $H_2O$ waste (17 to 24) 1,0 ml | $H_2O$ waste (17 to 24) 1,0 ml |
| $H_2O$ (cycles 9 to 16) 1,5 ml | Gel (cycles 9 to 16) 1,2 ml | Gel (cycles 9 to 16) 1,1 ml | NaOH (cycles 9 to 16) 1,5 ml | HCl (cycles 9 to 16) 1,5 ml | $H_2O$ (cycles 9 to 16) 1,5 ml | $H_2O$ (cycles 9 to 16) 1,5 ml | $H_2O$ waste (9 to 16) 1,0 ml | Gel (cycles 9 to 16) 1,1 ml | $H_2O$ waste (9 to 16) 1,0 ml | $H_2O$ waste (9 to 16) 1,0 ml | $H_2O$ waste (9 to 16) 1,0 ml |
| $H_2O$ (cycles 1 to 8) 1,5 ml | Gel (cycles 1 to 8) 1,2 ml | Gel (cycles 1 to 8) 1,1 ml | NaOH (cycles 1 to 8) 1,5 ml | HCl (cycles 1 to 8) 1,5 ml | $H_2O$ (cycles 1 to 8) 1,5 ml | $H_2O$ (cycles 1 to 8) 1,5 ml | $H_2O$ waste (1 to 8) 1,0 ml | Gel (cycles 1 to 8) 1,1 ml | $H_2O$ waste (1 to 8) 1,0 ml | $H_2O$ waste (1 to 8) 1,0 ml | $H_2O$ waste (1 to 8) 1,0 ml |

**Figure A.1 — Buffer tray configuration example[3)]**



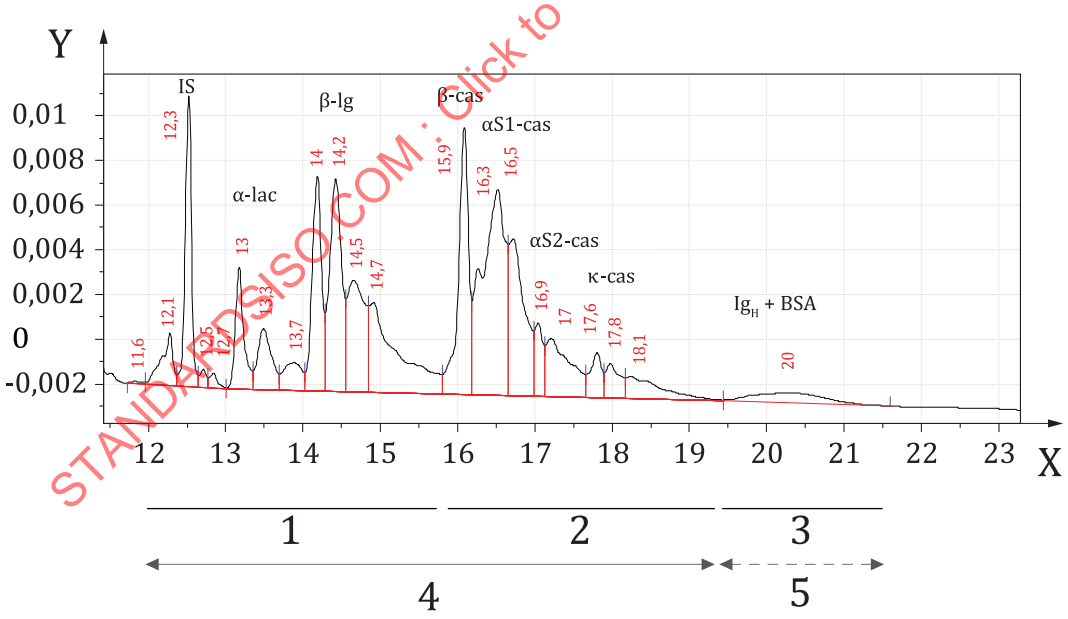
3) This layout is for the Beckman Coulter/Sciex PA 800 plus instrument. This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.




**Key**

| | | | |
|---|---|---|---|
| X | minutes | 3 | HMW whey |
| Y | AU | 4 | automatic integration |
| 1 | LMW whey | 5 | manual integration |
| 2 | caseins | | |

**Figure A.2 — Electropherogram of skim milk powder**




**Key**

| | | | |
|---|---|---|---|
| X | minutes | 3 | HMW whey |
| Y | AU | 4 | automatic integration |
| 1 | LMW whey | 5 | manual integration |
| 2 | caseins | | |

**Figure A.3 — Electropherogram of typical infant formula**