INTERNATIONAL STANDARD

ISO 17129

IDF 206

First edition
2006-07-01

Milk powder — Determination of soy and pea proteins using capillary electrophoresis in the presence of sodium dodecyl sulfate (SDS-CE) — Screening method

Lait en poudre — Détermination des protéines de soja et de pois par électrophorèse capillaire en présence de dodécyl sulfate de sodium (SDS-CE) — Méthode de criblage

Reference numbers
ISO 17129:2006(E)
IDF 206:2006(E)

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Published in Switzerland

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Foreword

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Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 17129|IDF 206 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

Introduction

The compositional standards on many milk products require that they contain no other proteins than milk proteins. However, despite the fact that the low prices of some non-milk proteins could make them attractive as potential adulterants, there are at present no standardized methods for their determination in milk powders and other dairy products.

The method in this International Standard has been developed with the following two objectives:

a) to cover the need to control the authenticity of products;

b) to assist, through its application, in controlling and restraining fraud.

The choice and the first assessment of the method were carried out within the EU project SMT4-CT97-2205 (IV Frame Work).

Milk powder — Determination of soy and pea proteins using capillary electrophoresis in the presence of sodium dodecyl sulfate (SDS-CE) — Screening method

1 Scope

This International Standard describes a method for the determination of the soy and pea protein isolates in low-heat milk powder, using capillary electrophoresis in the presence of sodium dodecyl sulfate (SDS-CE).

The method is not suitable for detecting the presence of hydrolysed plant proteins in milk powder.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 14891|IDF 185, *Milk and milk products — Determination of nitrogen content — Routine method using combustion according to the Dumas principle*

ISO 8968|IDF 20 (all parts), *Milk — Determination of nitrogen content*

3 Terms and definitions

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

3.1
soy and pea proteins
mass fraction of soy and pea proteins determined by the procedure specified in this International Standard

NOTE The soy and pea proteins are expressed as a mass fraction of the total protein content of the sample. The factor for calculation of total proteins of a test sample is 6,38 × N %, where N is the nitrogen content.

4 Principle

The milk proteins present in a test portion are removed selectively by using tetraborate EDTA buffer to enhance the detection of small amounts of added plant protein. A tris-HCl buffer is added in the presence of sodium dodecyl sulfate and a reducing agent to dissolve the precipitate, in order to dissociate proteins and disrupt any protein aggregates formed by S-S bonds. The proteins are separated and determined by capillary electrophoresis. The amount of plant proteins is quantified by previous calibration.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and double-distilled or demineralized water or water of equivalent purity.

5.1 Extraction buffer

Dissolve 1,14 g of disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) and 1,49 g of ethylene-diaminetetraacetic acid disodium salt dihydrate (EDTA; $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) in a measuring cylinder (6.1) in about 80 ml of water. Dilute with water to 100 ml and mix.

Check whether the pH of the extraction buffer is 8,3 ± 0,1. If the final pH value is outside that range, repeat the preparation of the extraction buffer while changing all reagents. The adjustment of pH by chemical agents is not acceptable.

5.2 Sample buffer

Dissolve 606 mg of tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$), 1,00 g of dodecyl sulfate sodium salt, (SDS; $C_{12}H_{25}O_4SNa$) and 37 mg of EDTA ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) in a measuring cylinder (6.1) in about 80 ml water and mix. Add 14,7 ml of hydrochloric acid with concentration c(HCl) = 0,1 mol/l, and 2 ml of 2-mercaptoethanol. Dilute with water to 100 ml and mix.

Check whether the pH of the sample buffer is 8,7 ± 0,1. If the final pH value is outside this range, repeat the preparation of the sample buffer while changing all reagents. The adjustment of pH by chemical agents is not feasible.

5.3 Electrophoresis buffer, for example Beckman eCAP™SDS 14-200 gel buffer[1)] or equivalent.

5.4 Sodium hydroxide solution, c(NaOH) = 0,1 mol/l.

5.5 Reference test mix, containing proteins with molecular mass between 10 kDa and 200 kDa.

NOTE The reference test mix can be obtained as a commercially available mixture.

5.6 Reference sample calibrant, with a known percentage of plant protein, supplied by NIZO (Ede, NL)[2)]. The protein content of the calibrant will also be known.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Graduated measuring cylinder, of capacity up to 100 ml.

6.2 Pasteur pipette.

6.3 Micro-vial, with screw cap, of capacity 1,5 ml.

6.4 Analytical balance, capable of weighing to the nearest 0,1 mg.

6.5 Centrifuge, capable of spinning with radial acceleration of up to 6 500 g.

6.6 pH meter, of minimum sensitivity 0,1 pH unit, with a glass electrode and a suitable reference electrode, with temperature compensation.

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1) Beckman eCAP™SDS14-200 gel buffer is an example of a suitable product available commercially.

2) Address: NIZO Food Research B.V., P.O. Box 20, 6710 BA, Ede, The Netherlands.

This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of the mentioned products.

6.7 Vortex mixer.

6.8 Thermomixer, Eppendorf 5436[3)] or similar.

6.9 Capillary electrophoresis instrument, with linear voltage gradient.

6.9.1 Columns, hydrophilically coated fused-silica capillary, of effective length about 20 cm (injector to detector) and internal diameter of 75 µm.

6.9.2 UV detector, capable of measuring at approximately 214 nm.

6.9.3 Software, capable of subtracting the blank value from the sample run.

6.10 Data system, capable of producing information required in Clauses 9 and 10.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50.

8 Preparation

8.1 Protein content of test sample

Determine the total protein content of the test samples by using the method specified in ISO 14891|IDF 185 or the method specified in the relevant part of ISO 8968|IDF 20.

8.2 Preparation of test portion

If the protein content of the test sample is between a mass fraction of 30 % and 40 %, weigh, to the nearest 0,1 mg, 126 mg of test sample in a micro-vial with screw cap (6.3). If the protein content of the test sample is outside this range, change the mass of the sample proportionally.

Add 1 ml of extraction buffer (5.1) to the test portion in the micro-vial. Mix the obtained solution with the vortex mixer (6.7) at about 2 500 r/min for 1,5 min. Allow to stand for 5 min then mix the solution again for 1,5 min. Centrifuge the mixture at 6 500 g for 30 min. Use a Pasteur pipette to carefully remove the supernatant from the residue.

Wash the residue with 1 ml of extraction buffer (5.1). Centrifuge the washed residue at 6 500 g for 20 min. Use a Pasteur pipette to carefully remove the supernatant. Wash the remaining residue once more by the same procedure.

Add 250 µl of sample buffer (5.2) to the washed residue in the micro-vial. Close the vial and heat it at 95 °C for 10 min while stirring with the thermomixer (6.8) set at about 1 000 r/min. Cool the vial in cold (ice) water or on ice. After cooling, centrifuge the vial and its contents again at 3 000 g for 5 min. Transfer about 200 µl of the clear supernatant to a suitable injection vial to use as the test solution.

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3) Eppendorf Thermomixer 5436 is an example of a suitable product available commercially.

This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of the mentioned product.

After heating, the sample in the micro-vial may also be stored in a freezer.

The above centrifuging step may be omitted if the contents of the micro-vial are already visually clear.

9 Procedure

9.1 Operating conditions

9.1.1 Running conditions for capillary electrophoresis (CE)

Reserve a capillary column (6.9.1) to be used for this analytical procedure only. If new, flush the column with water. Before each series of analyses, flush the used capillary with sodium hydroxide solution (5.4) for 0,5 min, followed by flushing with electrophoresis buffer (5.3) for 5 min.

Before each separation, flush the capillary again, preferably in the reversed direction, with electrophoresis buffer (5.3) at 241 325 Pa (35 psi) for 1 min.

Migrations are run at 25 °C. Start the electrophoresis with the capillary electrophoresis instrument (6.9) set at a voltage of 2 kV followed by a linear voltage gradient from 2 kV to 7 kV over 1,7 min. Then keep the voltage constant at 7 kV up to a total electrophoresis time period of 16 min. The current should be about 20 µA and the instrument shall be grounded at the injector side. Set the detector at 214 nm, the data collection at 2 Hz and the rise time at 0,5 s.

On the basis of instrumental characteristics, operators should adapt the operating conditions in order to obtain comparable qualitative separations.

9.1.2 Injection

Inject the test solution (8.2) at 3 447,5 Pa (0,5 psi) for 60 s. Then dip the injection side of the capillary in a vial of water for 6 s, followed by injection of the electrophoresis buffer (5.3) from the electrophoresis vial at 3 447,5 Pa (0,5 psi) for 5 s. Perform the migration after this step.

Do not use the capillary with other types of buffer. After use, flush the capillary with water. Store it with both ends in water. Depending on differences in pressure in the instrument, change the flushing and injection times accordingly.

9.2 Testing instrument suitability

Inject the reference test mix (5.5) three times. Check the consistency of the migration time and peak area of the proteins from the test mix. The difference obtained shall be less than 5 % (relative).

A new capillary might adsorb proteins, leading to increasing peak areas with repetitive injections of the same test sample. To avoid that, briefly flush the capillary with reference sample (8.2) in the normal direction for about 6 s. Then flush with electrophoresis buffer (5.3) in the same direction.

NOTE Deviations of the electropherograms can be caused by

— the capillary (including badly cut capillary inlet),

— insufficient temperature stability of the capillary (e.g. level of cooling liquid too low),

— deuterium lamp has deteriorated, or

— other instrument malfunction.

9.3 Qualitative analysis

9.3.1 Analysis

Analyse the test solution (8.2) under the operating conditions described in 9.1.

9.3.2 Peak identification

Identify the obtained peaks of the test sample by comparing them with the electropherograms shown in Figures A.1, A.2 and A.3. These electropherograms show peak patterns of genuine skimmed milk powder, and samples adulterated with soy and pea, respectively.

9.4 Quantitative analysis

9.4.1 Analysis

Analyse the test solution (8.2), the blank sample (9.4.2) and the reference samples (5.6) under the operating conditions as described in 9.1.

9.4.2 Blank sample

Inject the sample buffer (5.2) at 3 447,5 Pa (0,5 psi) for 50 s. The baseline obtained shall be relatively flat.

9.4.3 Measurements

Apply the following sequence for the measurement:

a) reference samples (5.6);

b) blank sample (9.4.2);

c) test solutions (8.2);

d) blank sample (9.4.2);

e) reference samples (5.6).

Repeat the sequence when the number of test samples is more than 10 to 15 samples.

9.4.4 Peak identification

Identify the obtained peaks of the test sample as described in 9.3.2. The electropherograms in Figures A.1 to A.3 show the peak patterns of genuine skimmed milk powder and samples adulterated with soy and pea, respectively.

9.4.5 Integration of peak areas

Adjust the integration parameters so that the integration is comparable to that shown in Figure A.2 or A.3 (draw baseline valley-to-valley). Do not group several peaks into one peak because that will cause inaccuracy in the calculation of the total peak area.

Calculate for each peak of protein (i) the normalized peak area, A_{ni}, by using the following equation:

$$A_{ni} = A_i / t_i$$

where

A_i is the numerical value of the peak area of protein i;

t_i is the numerical value of the migration time of peak i, in minutes.

For the pea proteins, use the sum of the normalized peak areas calculated separately.

NOTE Modern data acquisition software affords standard calculation of normalized peak areas.

9.4.6 Calibration

9.4.6.1 Reference sample

Preferably, use two reference samples (5.6) with a known percentage of plant protein in the required measuring range. Measure the reference samples as described in 9.4.3. Calculate their peak areas as described in 9.4.5.

9.4.6.2 Calculation of the response factors

Calculate the response factor, R_{fi}, for each calibrant (i) in the reference samples (5.6) by using the following equation:

$$R_{f\,i} = P_c / A_{ci}$$

where

P_c is the mass fraction of plant protein in the total protein of the calibrant, as a percentage (see 8.1);

A_{ci} is the numerical value of the normalized peak area of the reference calibrant (see 9.4.5).

If the response factors obtained for the calibrants, R_{fi}, differ by less than 5 %, use the calculated average response factor, R_f . Otherwise, calculate the linear regression constants for R_{fi}.

10 Calculation and expression of results

10.1 Qualitative analysis

Classify a sample as “suspect” if the electropherogram of the test sample (Clause 8) shows peaks being different from those of a genuine milk sample as shown in Figure A.1.

An electrophoretic pattern with peaks identified as in Figures A.2 and A.3 means the presence of soy or pea isolates, on the basis of the uniqueness of peak characteristics under the described analytical conditions.

Submit the electrophoretic pattern of “suspect” samples to the quantitative analysis procedure given in 10.2.

10.2 Quantitative analysis

10.2.1 Calculation

Calculate the mass fraction of adulteration of plant protein, P_s, as a percentage of total protein in the test sample by using the following equation:

$$P_s = R_f \times A_{nsi}$$

where

R_f is the numerical value of the average response factor (see 9.4.6.2);

A_{nsi} is the numerical value of the normalized peak area of the sample, obtained using the equation in 9.4.5.

When using the regression equation given in 9.4.6.2, substitute A_{nsi} and R_f in the equation and calculate P_s.

10.2.2 Expression of test results

Express the results to two decimal places.

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.

11.2 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

— protein content level of 0,99 %: r = 0,37 %

— protein content level of 1,96 %: r = 0,98 %

— protein content level of 4,76 %: r = 1,48 %

11.3 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

— protein content level of 0,99 %: R = 1,29 %

— protein content level of 1,96 %: R = 1,88 %

— protein content level of 4,76 %: R = 3,58 %

12 Test report

The test report shall specify:

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

b) the sampling method used, if known;

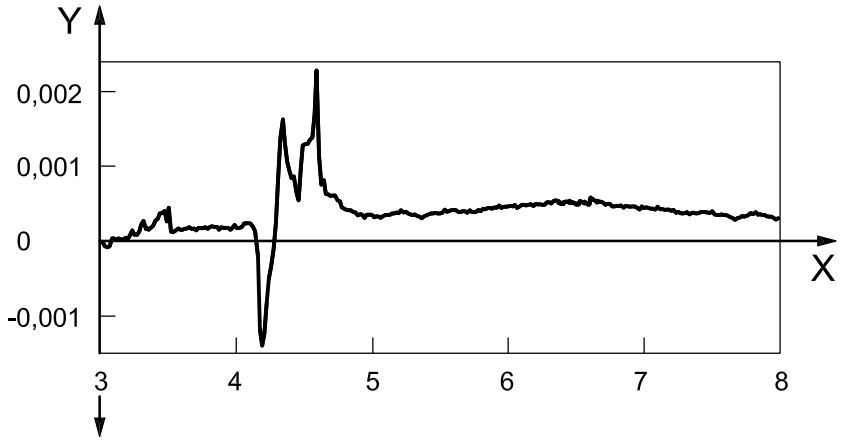
c) the test method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

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

Annex A
(informative)

Examples of electropherograms

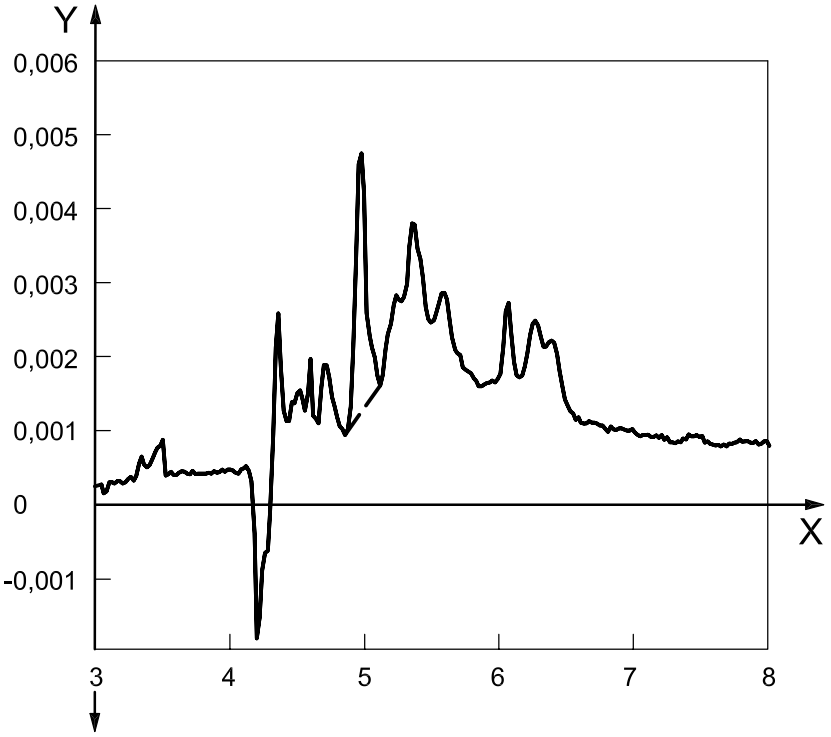


Key

X time, min

Y absorbance at 214 nm

Figure A.1 — SDS-CE electropherogram of genuine milk

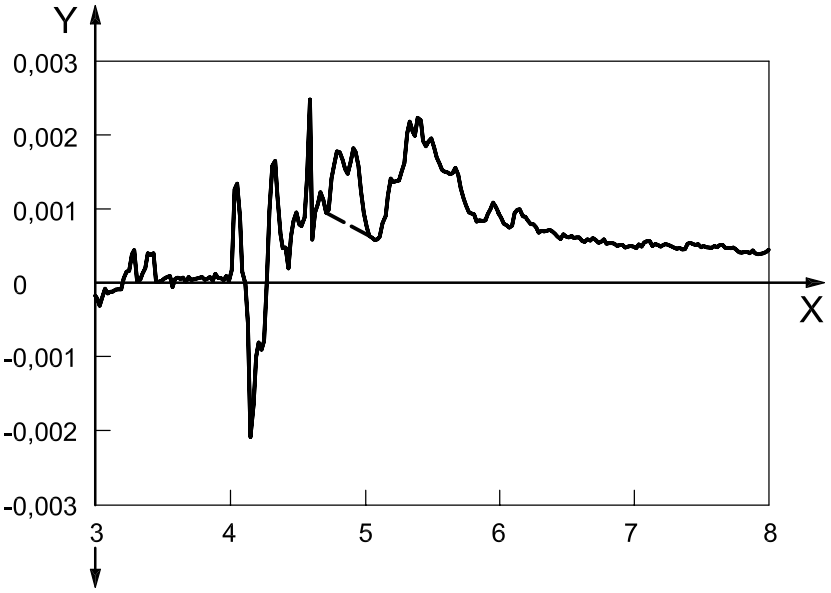


Key

X time, min

Y absorbance at 214 nm

Figure A.2 — SDS-CE electropherogram of milk powder with added soy protein; the base line indicates the peak to be quantified



Key

X time, min

Y absorbance at 214 nm

Figure A.3 — SDS-CE electropherogram of milk powder with added pea protein; the base line indicates the peaks to be quantified.

Annex B
(informative)

Interlaboratory trials

An international collaborative test involving eight laboratories was carried out on two different samples with three types of protein added in the same amount to low-heat milk powder. The thus-obtained test samples were divided again into a total of 22 blind duplicate samples. The calibration samples are shown in Table B.1. The test was organized by the Dairy Science Institute, Lodi, Italy. All values are expressed as mass fractions.

The results obtained were subject to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision data shown in Table B.2.

Table B.1 — Concentration of vegetable protein in soy and pea calibration standards (mass fraction of vegetable protein in total protein)

Mass fraction of vegetable protein in total protein %	
Soy calibration standards[a]	**Pea calibration standards**
0,52	0,48
1,05	0,94
2,08	1,87
4,07	3,69
7,82	7,11

[a] Containing a commercial soy protein isolate (type A).

Table B.2 — Results of the interlaboratory test on determination of added soy and pea proteins in low-heat milk powder by SDS-CE

	Blank	Protein A			Protein B			Protein C			
True values of added proteins, %	0,00	0,99	1,96	4,76	0,99	1,96	4,76	0,00	0,99	1,96	4,76
Number of laboratories retained after eliminating outliers	8	8	8	8	8	8	8	8	7[a]	7[a]	7[a]
Mean value, %	0,16	1,09	1,90	4,02	1,14	2,28	5,08	0,21	1,10	2,09	4,79
Repeatability limit, r (= 2,8s_r), %	0,06	0,47	1,10	1,68	0,39	0,93	1,63	0,22	0,25	0,92	1,13
Repeatability standard deviation, s_r, %	0,02	0,17	0,39	0,60	0,14	0,33	0,58	0,08	0,09	0,33	0,40
Coefficient of variation of repeatability, %	14	15	21	15	12	15	12	38	8	16	9
Reproducibility limit, R (= 2,8 s_R), %	0,44	1,31	2,16	3,71	1,26	1,63	3,48	0,70	1,30	1,85	3,56
Reproducibility standard deviation, s_R, %	0,16	0,47	0,77	1,32	0,45	0,58	1,40	0,25	0,46	0,66	1,27
Coefficient of variation of reproducibility, %	96	43	41	33	39	26	25	116	42	32	27

Explanation of protein used to prepare the adulterated milk powder:

Protein A = soy protein type A (commercial soy protein isolate);
Protein B = soy protein type K (commercial soy protein isolate);
Protein C = pea protein (commercial pea protein isolate).

[a] Values obtained after elimination of outliers.

The probabilities that the true mean difference between genuine powder milk and milk powder adulterated at 1 % was zero [$P(T \leqslant t)$], with 15 degrees of freedom, were respectively: $2{,}12 \times e^{-08}$ for protein A; $8{,}01 \times e^{-09}$ for protein B; and $4{,}17 \times e^{-07}$ for protein C.

Table B.3 — Mean results for repeatability and reproducibility limits

	True values of added proteins (%)		
	0,99	**1,96**	**4,76**
Repeatability limit, r			
Protein A, %	0,47	1,10	1,68
Protein B, %	0,39	0,93	1,63
Protein C, %	0,25	0,92	1,13
Mean repeatability limit, r, %	**0,37**	**0,98**	**1,48**
Reproducibility limit, R			
Protein A, %	1,31	2,16	3,71
Protein B, %	1,26	1,63	3,48
Protein C, %	1,30	1,85	3,56
Mean reproducibility limit, R, %	**1,29**	**1,88**	**3,58**

Bibliography

[1] ISO 707|IDF 50, *Milk and milk products — Guidance on sampling*

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

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ICS 67.100.01

Price based on 13 pages