FOOD COMPOSITION AND ADDITIVES

Determination of Vegetal Proteins in Milk Powder by Sodium Dodecyl Sulfate–Capillary Gel Electrophoresis: Interlaboratory Study

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An interlaboratory study, with the participation of 8 laboratories, was conducted to evaluate a sodium dodecyl sulfate–capillary gel electrophoresis method for determination of adulteration of milk powder with soy and pea proteins. Calibration standards (0–8%, w/w, soy and pea protein in total protein) and adulterated skim milk powders (0–5%, w/w, soy and pea proteins in total protein) were produced. Vegetal proteins were determined after removal of milk proteins by pretreatment of the samples with tetraborate–EDTA buffer, pH 8.3. Repeatability standard deviations ranged from 9 to 15% and reproducibility standard deviations ranged from 25 to 30% in the samples containing 5% vegetal protein in total protein.

The compositional standards of most dairy products require that they contain no proteins other than milk proteins. However, although the low cost of some vegetal proteins (soy, maize, pea, bean, rice, wheat, and potato) could make them attractive as potential adulterants, there are, at present, no international standards and routine methods to determine them in milk powders and other dairy products. This lack of control could be an incentive to commit fraud.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) has been used to study adulterations of dairy products with vegetal proteins such as soy, because it provides good separations and is not much affected by food processing, particularly heat treatment (1–3). However, results are difficult to quantitate and the technique is rather time-consuming. These drawbacks can be overcome with capillary electrophoresis (CE), which provides rapid and automated analysis, and is associated with a very high resolving power and accurate quantitation of the resulting data (4). Sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE), combined with a tetraborate–EDTA sample pretreatment (2), allowed detection of 1% soy protein in total protein in mixtures of milk powder and soy preparations (5).

In 1998, a European project within the Standards, Measurements and Testing Program was granted for the detection of nonmilk proteins in milk products (SMT4-CT97-2205). During the project, the suitability of using several commercial isolates and hydrolysates of vegetal proteins (soy, pea, wheat, and maize) to prepare adulterated test materials was assessed. Calibration standards from 0 to 8% added protein and skim milk powders adulterated with 0 to 5% added soy, pea, and wheat isolates and subjected to low and high heat treatments were produced. Various immunological and electrophoretic methods were evaluated for the detection of the fraudulent addition of these nonmilk proteins to milk powder, and 3 methods [SDS–PAGE and SDS–CGE, both combined with a tetraborate–EDTA pretreatment, and indirect competitive enzyme-linked immunosorbent assay (ELISA)] were selected for in-house prevalidation and adaptability tests. Finally, SDS–CGE and indirect competitive ELISA were chosen for final validation as quantitative methods through interlaboratory studies.

This paper describes the final validation of an SDS–CGE method to detect soy and pea proteins in low-heat milk powders through interlaboratory studies. The validation of ELISA methods is reported in a separate paper.

Interlaboratory Study

Test Materials

Calibration standards and milk powders (genuine and adulterated) were prepared at NIZO Food Research (Ede, The Netherlands) and distributed to all participating laboratories.

Preparation of Calibration Standards

Calibration standards were prepared with extra milk proteins to achieve the desired vegetal-protein-to-total-protein ratio in a reduced total volume in order to facilitate freeze-drying. For this purpose, 160 g skim milk powder (NILAC; NIZO Food Research) was dissolved in 1.6 L water at 40°C and stirred for 2 h. A milk protein solution was prepared by dissolving 23 g whey protein concentrate (Bipro; Davisco Food International, Le Sueur, MN) and 93 g sodium caseinate (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in 1.6 L water at 10°C, using mechanical stirring and ultrasonic treatment, and adjusting the pH to 7.8 with 4M NaOH. The reconstituted milk and the milk protein solution were mixed, giving a final pH of 7.1. Soy protein isolate A (5.62 g; Supro 500 E, 85%, w/w, protein; Anvisa, Madrid, Spain) and pea protein isolate (5.3 g; Pisane HD, 84%, w/w, protein; Cosucra, Fontenoy, Belgium) were each added to 1.2 kg of the protein-enriched milk to give 7.82 and 7.11% adulteration, respectively (w/w, vegetal protein/total protein). Standard solutions were homogenized twice at 500 bar and 45°C to prevent vegetal proteins from settling out. The homogenized solutions did not show any precipitated matter after a few days at 4°C. Dilutions were made on a weight basis to give 4.07, 2.08, 1.05, and 0.52% adulteration for soy proteins, and 3.69, 1.87, 0.94, and 0.48% for pea proteins. The solutions were then freeze-dried. Finally, the products were equilibrated with air containing about 50% humidity to minimize the hygroscopic character of the freeze-dried powder, resulting in a moisture content of about 10%.

Preparation of Adulterated Milk Powder Samples

Soy protein isolate K (Soy 595, 84.1%, w/w, protein; Europroducts, Milan, Italy) was used in addition to soy protein isolate A and pea protein isolate to assess the effect of the source of soy protein. Each vegetal protein preparation (0.82 kg) was added to 400 L skim milk, and the pH was adjusted to 7.2 with 6M NaOH. These solutions (4.76%, w/w, vegetal protein in total protein) were stirred overnight at 4°C and homogenized 3 times at 250 bar and 45°C. Dilutions were made with reconstituted milk to yield adulteration levels of 1.96 and 0.99% (w/w) vegetal protein in total protein. Adulterated samples were subsequently pasteurized and spray-dried using a pilot plant spray-drier at low-heat conditions, resulting in low-heat spray-dried powder. Blank samples were also prepared. The powders were packaged in tins and stored at –18°C.

Homogeneity and Stability Testing of Milk Powders

From each batch of milk powder, 10 tins were randomly selected. Each tin was sampled twice and analyzed by SDS–CGE for homogeneity testing. For stability studies, milk powders were stored at 40, 25, 7, and –20°C, and sampled (2 samples analyzed in duplicate) after 2 and 4 weeks, respectively.
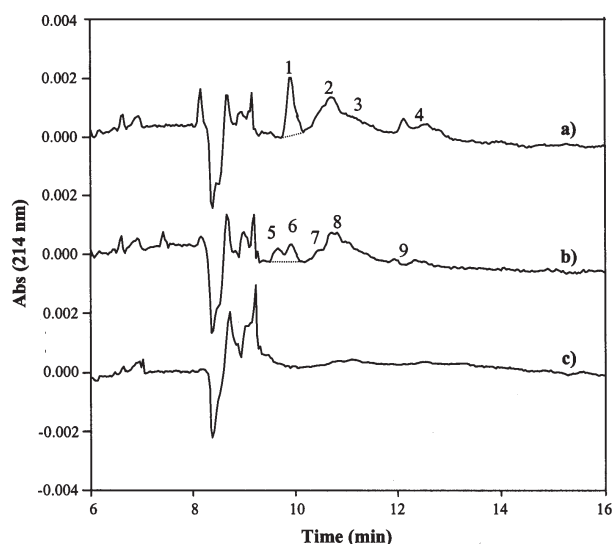

Figure 1. SDS–CGE electropherograms of calibration standards containing (a) 4.07% soy protein and (b) 3.69% pea protein in total protein, and (c) a blank low-heat milk powder sample, after extraction of milk proteins with tetraborate–EDTA buffer. (1) Glycinin B; (2) glycinin A; (3) β-conglycinin; (4) β-conglycinin α and α'; (5) legumin β; (6) legumin αm; (7) vicilin; (8) legumin αM; (9) convicilin.

Organization of the Interlaboratory Study

Two interlaboratory studies for the detection of soy and pea proteins in low-heat milk powder were performed by SDS–CGE. Eight laboratories from different European countries, representing a cross-section of food control, university, and industry affiliations, were involved. Contractors met at a precollaborative trial workshop, where problems experienced with sample analysis and details of the organization of the trials were discussed.

Seven different pasteurized milk powders were selected for validation of the soy protein assay (the blank and 3 different concentration levels of the 2 different soy protein preparations, A and K). For validation of the pea protein assay, the 3 concentration levels and the blank were tested. Two replicates of each addition level, as blind duplicate test samples randomly coded, were conducted to assess within-laboratory precision parameters. A single determination was performed per test sample. Each interlaboratory test, including analysis of calibration standards and samples, was conducted in 1 day. All participants were strongly encouraged to follow the instructions established in the method and interlaboratory study protocols supplied with the samples. Data were processed following AOAC recommendations for design of a collaborative study (6).

METHOD

(Applicable to determine amounts ≥1% soy or pea protein/total protein in low-heat milk powder samples.)

Principle

Milk proteins are soluble in tetraborate–EDTA buffer, pH 8.3, whereas vegetal proteins precipitate. This allows the selective removal of milk proteins, enhancing the detection of low proportions of vegetal protein added. Precipitated vegetal proteins are dissolved in a Tris–HCl buffer in the presence of SDS and a reducing agent, in order to dissociate proteins and disrupt any protein aggregates formed by S–S bonds. The amount of vegetal protein is determined by separating the proteins by SDS–CGE and quantitation of the selected peaks.

Apparatus

(**a**) *Centrifuge*.—Capable of generating minimal $6500 \times g$.

(**b**) *pH Meter*

(**c**) *Analytical balance*.—0.1 mg accuracy.

(**d**) *Screw-capped Eppendorf vials*.—1.5 mL.

(**e**) *Vortex mixer*

(**f**) *Thermomixer*.—Eppendorf 5436 or equivalent; 95°C.

(**g**) *CE instrument*.—Equipped with UV detection at 214 nm and peak integration software.

(**h**) *Hydrophilic-coated fused-silica capillary*.—Supelco Select P1 (Bellefonte, PA) or equivalent; 20 cm effective length (injection to detector) id, 75 μm.

Reagents

All reagents were of recognized analytical grade. Water was double-distilled or of equivalent purity.

(**a**) *Disodium tetraborate decahydrate, $Na_2B_4O_7 \cdot 10H_2O$*.—Merck (Darmstadt, Germany).

(**b**) *EDTA disodium salt dihydrate, Titriplex®III, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$*.—Merck.

(**c**) *Tris(hydroxymethyl) aminomethane, TRIZMA®, $C_4H_{11}NO_3$*.—Sigma (St. Louis MO).

(**d**) *SDS, $C_{12}H_{25}NaO_4S$*.—Merck.

(**e**) *2-Mercaptoethanol*.—Merck.

(**f**) *HCl*.—0.1 mol/L.

(**g**) *Gel buffer*.—eCAP™ SDS 14-200 Beckman Instruments (Fullerton CA).

(**h**) *Test mix of proteins*.—With molecular mass from 10 to 200 kDa.

Sample Preparation

An extraction buffer (pH 8.3 ± 0.1) containing 1.14 g sodium tetraborate and 1.49 g EDTA in 100 mL water was used. Tetraborate–EDTA buffer (1 mL) was added to 126 mg milk powder (35% total protein) or to 75 mg calibration standard (60% total protein) and mixed on a Vortex mixer twice at 2500 rpm × 1.5 min, in a 1.5 mL screw-capped vial, with a 5 min interval between each blending. After centrifugation at $6500 \times g$ for 30 min, the supernatant was carefully removed with a Pasteur pipet, and the precipitate (extraction residue) was washed twice more with 1 mL tetraborate–EDTA buffer.

Electrophoresis sample buffer (pH 8.7 ± 0.1) was prepared by dissolving 606 mg Tris(hydroxymethyl) aminomethane, 1.00 g SDS, 37 mg EDTA, 14.7 mL 0.1M HCl, and 2 mL 2-mercaptoethanol in 100 mL water. Sample buffer (250 μL) was added to the extraction residue in the vial, and heated for 10 min at 95°C, with stirring at 1000 rpm. Samples were cooled in cold water and centrifuged at $3000 \times g$ for 5 min, and the clear supernatant was used for SDS–CGE analysis. Once heated, samples may be kept in the freezer.

Table 1. Interlaboratory study results for determination of soy protein in low-heat milk powder by SDS–CGE

Sample		Soy protein, % per total protein (experimental)							
Code	Soy, %	1	2	3	4	5	6	7	8
Blank	0.00	0.30	0.08	0.19	0.14	−0.02	0.00	0.37	0.37
Blank	0.00	0.15	0.08	0.16	0.06	−0.02	0.00	0.37	0.37
197	0.99 (A)	1.05	0.48	0.92	1.43	0.50	0.9	1.33	1.75
781	0.99 (A)	1.55	0.70	0.93	0.88	0.70	1.07	1.48	1.84
425	1.96 (A)	2.29	1.46	1.37	1.94	1.00	2.04	2.43	1.83
666	1.96 (A)	1.59	1.45	2.36	1.49	0.82	2.38	3.54	2.48
173	4.76 (A)	3.43	2.17	5.22	3.72	2.78	5.52	5.22	4.19
568	4.76 (A)	5.90	2.04	4.49	3.70	3.6	3.95	4.90	3.47
245	0.99 (K)	2.20	0.57	1.10	0.87	1.39	1.19	1.17	0.99
432	0.99 (K)	1.70	0.48	0.98	0.84	1.29	0.88	1.35	1.24
859	1.96 (K)	2.30	1.41	1.71	2.11	2.23	2.17	2.68	2.08
346	1.96 (K)	3.27	1.64	2.19	2.67	2.97	2.54	2.75	1.75
337	4.76 (K)	4.33	3.44	4.94	4.61	6.01	6.41	5.80	4.69
465	4.76 (K)	6.33	3.37	5.41	4.71	7.12	4.18	5.45	4.42

Table 2.   Interlaboratory study results for determination of pea protein in low-heat milk powder by SDS–CGE

Sample		Pea protein, % per total protein (experimental)							
Code	Pea, %	1	2	3	4	5	6	7	8
Blank	0.00	−0.11	0.59	0.06	0.61	0.28	0.00	0.28	−0.03
Blank	0.00	0.17	0.59	0.26	0.18	0.27	0.00	0.28	−0.03
543	0.99	1.50[a]	1.23	0.78	1.38	0.46	1.80	1.11	0.65
188	0.99	0.91	1.38	1.00	1.44	0.44	1.91	1.22	0.86
777	1.96	2.06	2.39	2.10	4.99[a]	1.69	2.77	2.09	1.41
123	1.96	1.55	3.30	1.98	1.07[a]	1.08	2.51	2.08	2.23
987	4.76	4.75	2.34	5.11	5.71	4.14	5.73	4.41	6.12
765	4.76	3.04	3.44	5.35	5.29	4.31	6.50	4.43	5.97

[a]   Data not included in the statistical analysis.

Determination by SDS–CGE

Before each separation, the capillary was flushed, preferably in the reversed direction, with the electrophoresis buffer for 1 min at 35 psi. Sample solution was injected for 60 s at 0.5 psi; this was followed by a dip of the injection side of the capillary in a vial of water for 6 s and hydrodynamic injection of the electrophoresis buffer (from the electrophoresis vial) for 5 s at 0.5 psi.

Migrations were run at 25°C, starting at 2 kV, followed by a linear voltage gradient from 2 to 7 kV in 1.7 min, and then a constant voltage of 7 kV with ground at the injector side (16 min total electrophoresis time, 20 μA approximate current). The detector was set at 214 nm (data collection at 2 Hz, rise time 0.5 s). Upon storage, the capillary was flushed with water.

The use of a test mix of proteins with molecular mass ranging from ca 10 to 200 kDa was recommended for testing instrument suitability.

Calculations

Normalized peak areas were calculated with the following equation:

$$A_{pi} = a_{pi}/t_{pi}$$

where A_{pi} = normalized peak area of protein i; a_{pi} = peak area of protein i; t_{pi} = migration time of peak i.

Linear regression lines were built up using calibration standards:

Table 3.   Statistical analysis of data from the interlaboratory study for determination of soy protein in low-heat milk powder by SDS–CGE

Soy, %[a]	Average	r[b]	R[c]	s_r[d]	s_R[e]	RSD$_r$[f]	RSD$_R$[g]	Rec., %[h]
0.00	0.16	0.06	0.44	0.02	0.16	14	96	—[i]
0.99 (A)	1.09	0.47	1.31	0.17	0.47	15	43	94
1.96 (A)	1.90	1.10	2.16	0.39	0.77	21	41	89
4.76 (A)	4.02	1.68	3.71	0.60	1.33	15	33	81
0.99 (K)	1.14	0.39	1.26	0.14	0.45	12	39	99
1.96 (K)	2.28	0.93	1.63	0.33	0.58	15	26	108
4.76 (K)	5.08	1.63	3.48	0.58	1.24	12	25	103

[a]   Two types of soy protein (A and K) were used to prepare the adulterated milk powders.
[b]   r = Repeatability.
[c]   R = Reproducibility.
[d] s_r = Repeatability standard deviation.
[e] s_R = Reproducibility standard deviation.
[f]   RSD$_r$ = Repeatability relative standard deviation.
[g]   RSD$_R$ = Reproducibility relative standard deviation.
[h]   Average recovery.
[i]   — = Not calculated.

Table 4. Statistical analysis of data from the interlaboratory study for determination of pea protein in low-heat milk powder by SDS–CGE

Pea, %	Average	r^a	R^b	$s_r{}^c$	$s_R{}^d$	$RSD_r{}^e$	$RSD_R{}^f$	Rec., %[g]
0.00	0.21	0.23	0.70	0.08	0.25	38	116	—[h]
0.99	1.10[i]	0.25[i]	1.30[i]	0.09[i]	0.46[i]	8[i]	42[i]	90[i]
1.96	2.09[i]	0.92[i]	1.85[i]	0.33[i]	0.66[i]	16[i]	32[i]	96[i]
4.76	4.79	1.13	3.56	0.41	1.27	9	27	96

[a]   r = Repeatability.

[b]   R = Reproducibility.

[c] s_r = Repeatability standard deviation.

[d] s_R = Reproducibility standard deviation.

[e] RSD_r = Repeatability relative standard deviation.

[f] RSD_R = Reproducibility relative standard deviation.

[g]   Average recovery.

[h]   — = Not calculated.

[i]   Values obtained after removal of outliers found by Cochran test.

$$A_{ci} = S_c \times P_c$$

where A_{ci} = normalized peak area of protein i in the calibration standards; S_c = slope of the regression equation; P_c = percentage of plant protein in total protein of the calibration standards.

The percentage of adulteration of the samples was calculated with the following equation:

$$P_s = A_{si}/S_c$$

where P_s = percentage of plant protein in total protein of sample s; A_{si} = normalized peak area of protein i in sample s; S_c = slope of the regression equation.

Results and Discussion

Homogeneity and Stability of Milk Powders

Replicate analyses of the samples of milk powder taken from each batch indicated that the samples were homogeneous within the precision of the SDS–CGE method. Regarding stability, a slight decrease in the vegetal protein content was observed in samples kept at 35 and 40°C after 4 weeks of storage.

Interlaboratory Study

Figure 1 shows the SDS–CGE electropherograms of calibration standards containing approximately 4% soy and pea proteins in total protein, after extraction with tetraborate–EDTA buffer. The electropherogram of a genuine sample obtained under the same conditions is also shown for comparison. Peaks used for quantitation corresponded to basic subunits of glycinin and to β and αm subunits of legumin, respectively, as identified by comparison with published SDS–PAGE patterns of soy (7, 8) and pea proteins (9, 10). These peaks were selected because they provided the best repeatability.

No negative comments were received from any of the participating laboratories. In some cases, depending on the

pressure and gradient possibilities provided by the CE instruments available in the different laboratories, the flushing, injection times, or running conditions had to be slightly modified. Also, if a longer capillary length was required, the field strength (kV/cm) was maintained by increasing the voltage.

Calibration lines, obtained from the analysis of the calibration standards, showed good linearity in the range of addition considered. Only one laboratory obtained a square correlation coefficient (R^2) < 0.99. The individual values obtained by the 8 participating laboratories in the determination of soy and pea protein in milk powder samples are reported in Tables 1 and 2, respectively. The results from the statistical analyses are shown in Tables 3 and 4. In general terms, the experimental values were reasonably close to the theoretical values. Adulterated materials were different from blanks in all cases and, within each laboratory, the 3 adulteration levels were well characterized. Average recoveries were between 81 and 108%. The Cochran test, which allows the removal of laboratories showing a significant variability among replicate analyses (6), revealed outlier values in pea data. Rejection of the outliers improved the precision of the results obtained for milk powders containing 0.99 and 1.96% pea protein in total protein (Table 4). The Grubbs test, for removal of laboratories with extreme averages (6), did not reveal outliers.

This study shows that SDS–CGE allows quantitative determination of the fraudulent addition of at least 1% soy and pea protein, expressed in total protein, to low-heat milk powder. We have found no literature reports on the detection of pea proteins added to milk products. Regarding the investigation of soy proteins, previous papers indicate low detection limits, but do not provide full quantitative data (1–3, 5). Recently, a reversed-phase liquid chromatographic method that allows quantitative determination of soy protein in milk, with a detection limit of 13 µg protein/g milk, was published (11). In those cases, milk samples spiked with soy proteins were used for the analysis. In the present work, samples adulterated at origin and subjected to different technological treatments such as ho-

mogenization, heat treatment, and spray drying, were studied. These treatments have an important negative effect on the separation between vegetal and milk proteins that can dramatically reduce recovery and highlight the need for very sensitive methods of analysis such as CE.

Tables 3 and 4 show that repeatability and reproducibility relative standard deviations of the CE–CGE method ranged between 8–21 and 25–43%, respectively. The low reproducibility is probably due to differences among CE equipment used in the various laboratories. Our results compare favorably with the few data found in the literature on the use of CE in interlaboratory studies, thus suggesting that this problem can be overcome with improvements in instrument standardization. Mopper and Sciacchitano (12) attributed the poor reproducibility of the results of an interlaboratory study on the determination of histamine in tuna by capillary zone electrophoresis (CZE) to interlaboratory variations in detector sensitivity, sample loading, voltage, and temperature control. Similar results were found in a study to identify wheat varieties by CZE (13).

Acknowledgments

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