

ELISA Kit for Casein Determination: Interlaboratory Study

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An interlaboratory study was performed in eight laboratories to validate an ELISA method developed for quantitative determination of casein in foods. The ELISA kit used is based on rabbit polyclonal antibodies. The kit is quite specific; no false-positive results or cross-reactivities were obtained for a broad range of food matrixes with zero content of milk proteins. All participants in the study received the casein kit, which included a standard operating procedure, a list of the samples, the samples, and a protocol for recording test results. The study included nine food samples: wheat flour, buckwheat flour, instant potato purée with milk, instant coffee with sugar and cream, a mixture for fancy bread, salami, liver paté, chocolate muesli with nuts, and a mixture for gluten-free bread. Three food samples with zero content of milk proteins showed a casein content lower than the lowest casein standard (1.0 mg CAS/kg) in most laboratories and measurements (64%). In 98% of the cases, the casein content was lower than the estimated LOQ. Two food samples with no dairy ingredient declared on the ingredient list contained casein levels higher than the second casein standard (3.0 mg CAS/kg) and the third standard (10.0 mg CAS/kg), respectively. Four food samples containing milk as an ingredient tested positive, and three showed casein contents higher than the highest standard (30.0 mg CAS/kg). The statistical tests (Cochran, Dixon) and analysis of variance were used for evaluation of the interlaboratory study results.

Repeatability and reproducibility limits as well as LOQ (1.8 mg CAS/kg) and LOD (0.5 mg CAS/kg) for the kit were calculated from the results of the interlaboratory study.

Milk is one of the main foods that can cause allergic reactions in children at early ages. However, even if the majority of children outgrow their allergy, milk allergy may persist even among adults (1–3). Prevalence figures for milk allergy differ depending on countries, but relevant population data varies between 1 and 3%. Cow's milk contains 30–35 g protein/L. Acidification of milk to pH 4.6 causes formation of two fractions: the whey fraction, which constitutes about 20% of the proteins, and the casein fraction (curd), which constitutes 80% of the milk proteins. The whey contains mainly beta-lactoglobulin (BLG), alpha-lactalbumin, bovine serum albumin, lactoferrin, and immunoglobulins. The curd consists of casein (CAS), occurring in four modifications, named α_{s1} , α_{s2} , beta, and kappa caseins. Some reports have shown that the caseins appear to be the major allergens responsible for milk allergy (4). Caseins are very susceptible to all proteinases and exopeptidases. To a lesser extent, they are affected by severe heat treatment. Multisensitizations to the different caseins most often occur in patients sensitized to the whole casein. The resulting allergenicity depends on temperature, heating time, and possible interactions with the food matrix (3, 5–11). There are only limited data on threshold levels of casein available from clinical studies using double-blind, placebo-controlled food challenge. In published reports of breast-fed babies experiencing severe allergic reactions, the concentration of cow's milk proteins was as low as a few ng/mL of milk, ranging from 0.5 to 50 ng/mL. Reactions often occurred at around 5 ng/mL (3).

Table 1. Samples studied

Sample	Description of sample	Milk content ^a
1	Wheat flour	N
2	Wheat flour spiked with casein (20 mg/kg)	S
3	Wheat flour spiked with casein (5 mg/kg)	S
4	Buckwheat flour	N
5	Buckwheat flour spiked with casein (20 mg/kg)	S
6	Buckwheat flour spiked with casein (5 mg/kg)	S
7	Instant potato purée with milk	Y
8	Instant coffee with milk and sugar (3 in 1)	Y
9	Mixture for cupcake	Y
10	Salami	N
11	Liver pate	Y
12	Chocolate muesli with nuts	N
13	Gluten-free mixture for bread	N
CS 1	Negative sample (part of the kit)	N
CS 2	Positive sample (part of the kit)	Y

^a N = Producer did not declare any milk content in the sample. Y = The content of milk or dairy compounds was declared on the label.
S = The spiked samples prepared by authors of the study.

For the detection and identification of cow's milk proteins, various analytical methods have been used, e.g., ELISA, chromatographic methods, electrophoretic methods, immunoblotting, and MS (12–14). Several commercial ELISA kits for casein determination are available. The influence of heat treatment on casein immunoreactivity and assay applicability was also tested (15). A newly published method (16) for casein quantification uses optical biosensor chip and gold nanoparticles based on the resonance enhanced absorption effect.

According to European Union (EU) directive 2003/89/EC and the Czech national legislation, designed ingredients with allergenic effect are subject to labeling rules. This means that all food producers have to provide appropriate information to consumers who suffer from food allergy. The aim of the EU food legislation is to provide the allergic consumer with safer food. The regulation should also result in a more efficient labeling control by official authorities. New and validated analytical methods are needed in this respect (3, 17–19).

Experimental

Principle of Determination

The determination of casein is based on its immunochemical reaction with specific antibodies. Casein present in samples and casein marked with biotin before analysis compete in the first step (incubation) in binding to specific antibodies coated on the walls of a microtiter plate. After the washing steps, horseradish peroxidase conjugated with streptavidin is added to the wells in an incubation phase. After the necessary incubation period and washing, addition

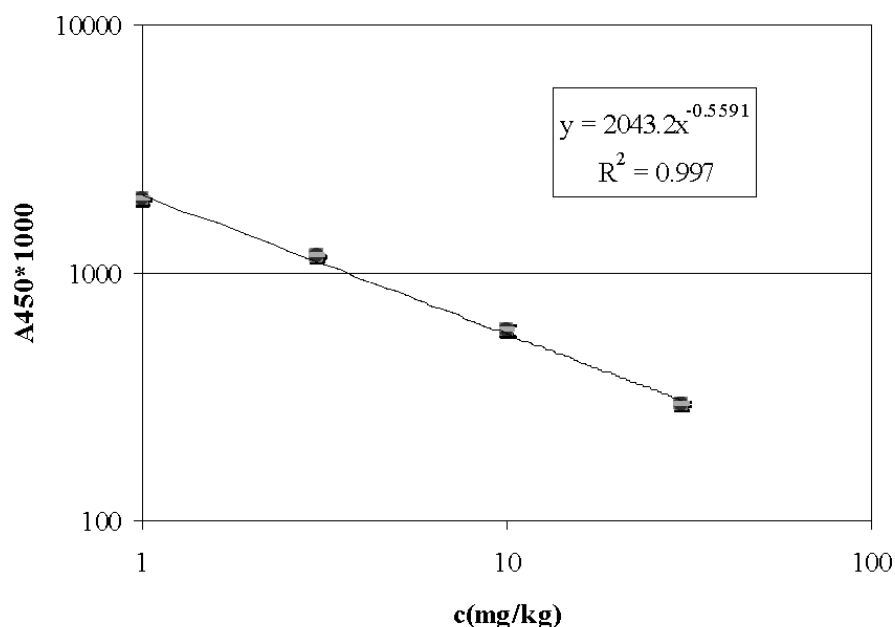
of a chromogenic substrate enables detection of the remaining immobilized peroxidase. The intensity of the color thus developed is inversely proportional to the concentration of casein in standard solutions, control samples, and analyzed samples.

Samples

The selected foods containing either declared milk protein (instant potato purée with milk, instant coffee with milk and sugar, mixture for cupcake, liver paté) or foods without any milk protein (wheat flour, buckwheat flour, salami, chocolate muesli with nuts, gluten-free mixture for bread) were purchased at the supermarket. Salami and chocolate muesli with nuts were homogenized with a rotor-speed mill (IKA Works, Inc., Wilmington, NC). The spiked samples of wheat flour and buckwheat flour were prepared by mixing 10 g casein into 90 g of the flour. This was further diluted with flour to final concentrations of 5 and 20 mg of CAS/kg of flour, respectively. The food samples were distributed together with the ELISA kit for the interlaboratory study. The list of the samples is shown in Table 1.

Chemicals

Casein ELISA kit.—Cat. No. FA 00208; SEDIUM RD, Ltd, Czech Republic. The format of the newly developed ELISA kit for casein determination in food and raw materials is a competitive assay based on polyclonal antibodies produced in a rabbit (AbkemIberia, 36202 Vigo, Spain; Cat. No. A1015). The antibodies were used for solid-phase coating. Casein from bovine milk (Sigma-Aldrich, Inc., St. Louis, MO; Cat. No. C5890) was used for preparation of standard solutions and for



casein (mg/kg)	A450*1000	SD
1	1 961	18.5
3	1 156	18.0
10	583	30.0
30	294	6.5

Figure 1. Calibration curve of casein, ranging from 1.0 to 30.0 mg CAS/kg.

conjugation with NHS-biotin (Roche Diagnostics GmbH, Mannheim, Germany; Cat. No. 34250). Streptavidin conjugate with horseradish peroxidase (Sigma-Aldrich; Cat. No. S5512) was used as a signal reagent. 3,3',5,5'-Tetramethylbenzidine liquid substrate (TMB) was used as substrate for ELISA (Sigma-Aldrich; Cat. No. T5513). The substrate is supplied as a one component ready-to-use solution.

ELISA Kit

The kit contains:

Microtiter plate with lid.—96 wells (arranged in 12 strips, each containing eight wells), inner walls of which are coated with the specific antibodies to casein (ready-for-use).

Standards (ready-for-use).—Six vials, each containing 0.5 mL of standard solutions. The vials contain casein concentrations of 0, 1.0, 3.0, 10.0, and 30.0 mg/kg, respectively; 0, 0.1, 0.3, 1.0, and 3.0 g CAS/mL, yellow color.

Control samples (CS1-negative: 0 g CAS/mL; CS2-positive: 0.5 g CAS/mL).—Two vials, each containing 0.5 mL, yellow color (ready-for-use).

Biotinylated casein.—NHS-biotin from Roche and alkaline buffer were used for the preparation of casein labeled by biotin.

Extraction buffer solution.—Two bottles of 300 mL content (ready-for-use).

Concentrate of conjugate (80 x).—One vial, containing 0.4 mL (concentrated conjugate of streptavidin with peroxidase in phosphate buffer solution containing stabilizers).

Buffer solution for dilution of conjugate.—Phosphate buffer solution, pink color; one bottle containing 24 mL (ready-for-use).

Wash solution concentrate (20 x).—Phosphate buffer with detergents; one bottle containing 50 mL concentrate.

TMB substrate.—One bottle containing 22 mL (ready-for-use).

Stop solution.—One vial containing 5.5 mL 2 M solution of hydrochloric acid (ready-for-use).

One vial for conjugate dilution.

Assay Parameters—In-House Validation

In-house validation of the kit was performed for setting certain assay parameters. The following parameters were determined:

Analytical sensitivity.—The standard (0.10 g/mL) was diluted twice and four times with the zero standard so that solutions with concentrations of 0, 0.025, 0.050, and

Table 2. Content of casein (mg/kg) in the samples (average of two replicates)

Sample	Laboratory							
	A	B	C	D	E	F	G	H
CS1 ^a	0.93	0.67	NA ^b	0.72	0.80	0.68	0.71	0.69
CS2	4.47	4.23	4.56	4.73	3.61	4.41	4.68	4.20
1 ^a	0.83	0.84	0.74	1.12	2.07	0.92	2.66	0.93
2	5.30	13.8	20.7	30.0	8.0	16.1	17.0	14.44
3	1.58	1.41	2.02	11.72	5.18	28.97	4.83	2.31
4 ^a	0.61	0.75	0.53	1.06	1.26	0.84	1.09	1.97
5	2.97	11.1	6.0	23.7	15.1	19.3	12.0	15.4
6	3.89	5.01	2.77	5.01	3.68	2.92	4.88	9.81
7 ^c	77.7	132.0	107.4	250.8	292.5	3479	2311	135.3
8 ^c	75.5	109.9	65.8	188.2	199.0	1193	879.1	128.1
9	12.6	14.6	12.0	22.0	27.0	22.8	15.6	15.3
10	7.22	7.38	4.26	12.7	11.1	12.9	11.5	7.05
11 ^c	31.1	55.3	33.6	88.2	108.8	159.4	104.1	35.1
12	9.3	11.0	7.4	23.4	23.5	33.5	13.5	17.0
13 ^a	0.58	0.68	0.98	0.64	0.89	0.86	0.91	0.85

^a Data only for information. At least 50% of results (in italics) were below the lowest calibration level.

^b N.A. = Data are not available.

^c Data only for information. All results (in italics) were above the highest calibration level.

0.10 g/mL were obtained. These solutions were measured 10 times (the zero standard 20 times) and the analytical LOD from the in-house validation (LOD_{IH}) was calculated. The LOD_{IH} value determined by this procedure was 0.009 g CAS/mL.

Functional sensitivity.—Defined addition of casein to 10 naturally dairy-free matrixes increased its content to the levels of 1.07–3.8 mg/kg. The value 0.1 g CAS/mL was determined as a reliable value that can be established as an LOQ from in-house validation (LOQ_{IH}).

Repeatability.—Intra-assay variance of the kit was tested on extracts of three dairy-free samples with defined addition of casein in 25 multiplicates. The maximum coefficient of variation (CV) was 18.6%. Interassay variance of the kit was determined on extracts of three dairy-free samples with defined addition of casein in 10 runs. The maximum CV was 16.2%.

Extraction effect.—Two food samples with natural contents of dairy ingredients were extracted five times and analyzed in one run. The differences between extractions, expressed as CV, were <11%.

Trueness.—**Dilution effect.**—Sets of diluted samples were prepared using the dilution buffer and extracts of three real food samples with natural contents of casein. All samples were analyzed in one run. Four levels of dilution were tested: 1:1, 1:3, 1:7, and 1:15. Recovery was in the range of 94.5–123.0%; thus, all these dilutions may be recommended for dilution of the samples with a high content of casein.

Recovery.—Defined addition of casein to three naturally dairy-free matrixes increased its content to levels of 4 and 8 mg CAS/kg, respectively. Recovery was in the range of 67.0–81.0%.

Zero matrixes.—The analysis included the following matrixes: raw materials for foodstuffs, the nature of which should justify the expectation of a zero content of milk proteins—wheat flour, fish meat, chicken meat, beef, pork, rice, sugar, ketchup, cocoa, syrup, vegetable oil, and eggs. Analysis detected no content of casein in any of the analyzed matrixes. The processed food samples containing milk or dairy components were tested as well, for example, ultra high temperature milk, milk powder, various kinds of cheese (fresh cheese, hard or processed cheese), and dairy desserts. All samples revealed the presence of casein.

Table 3. Classification of samples into categories

Category	Samples
1a	1, 4, 13, CS1
1b	7, 8, 11
2a	9, 10, 12
2b	2, 3, 5, 6, CS2

Table 4. Contingency table of false-positive and false-negative statements^a

	TRUE		FALSE		F/T ratio
Positive sample	TP	88	FN	0	0
Negative sample	TN	28	FP	3	0.11

^a Decision limit for FN and FP used was LOQ 1.8 mg/kg.

Interlaboratory Study

Eight laboratories with routine ELISA experience were included in the interlaboratory study. The participants received the casein kit with instructions together with 13 samples. They were asked to perform duplicate analyses of the samples under repeatability conditions, including two independent extracts and two measurements. The absorbance values from participants were required as well as remarks on technical conditions (washing procedure, ELISA reader, and centrifugation).

Sample Treatment

Laboratories received 5 g of each food sample and spiked sample. One gram of the sample was weighed and 10 mL of extraction buffer was added. The sample was shaken for 5 min and immediately centrifuged at 1800 g for 20 min. The supernatant was directly used for the assay without further dilution.

Determination

A 200 L volume of buffer for the first incubation was added to each well, and then 20 L of the standards, control samples, or samples was added to the corresponding wells. The plate was covered and incubated for 1 h at room temperature without shaking. The wells were washed four times with 300 L wash solution using a washer or repeating dispenser. Conjugate working solution (200 L) was immediately dispensed into all the wells.

The plate was covered and incubated for 1 h at room temperature without shaking. The contents of all wells were washed four times with 300 L wash solution using a washer or a repeating dispenser. TMB substrate (200 L) was immediately added to all the wells. The plate was incubated for 20 min in the dark without shaking. A 50 L volume of stop solution was added, and the plate was shaken briefly and read within 15 min at 450 nm.

Calculations

A calibration graph was constructed from the measured absorbance of the corresponding calibration solutions subtracted by the absorbance of zero standard (vertical axis – logarithmic scale) versus casein concentration in the solution (horizontal axis – logarithmic scale). A power equation was used for graph construction. An example of a calibration curve is shown in Figure 1.

Results and Discussion

Sample absorbance values obtained from participating laboratories were evaluated by Excel software. The calculated results of all samples in the interlaboratory study are presented in Tables 2 and 3. Three samples of food with zero content of milk proteins showed a casein content lower than the lowest standard (1.0 mg CAS/kg). Two samples of food with no dairy ingredient declared on the label had casein contents higher than the second standard (3.0 mg CAS/kg) and the third standard (10.0 mg CAS/kg), respectively. Four food samples containing milk as an ingredient were positive; three of them had casein contents higher than the highest standard (30.0 mg CAS/kg).

Statistical Evaluation

Samples included in the test were classified in two types:

(1) *Samples out of scope of the kit.*—(a) *Negative samples.*—Samples without casein content and lower than the lowest standard in 50% of laboratories. These samples were not assessed in a quantitative way, and the concentration values are only roughly informative. (b) *Highly positive samples.*—Samples with casein contents higher than the highest point of the calibration curve in the majority of the participating laboratories. These samples were not assessed in a quantitative way, and the concentration values are only roughly informative.

(2) *Samples within the quantification range, i.e., findings between the lowest and the highest standard.*—(a) *Naturally positive samples.*—With dairy ingredient. (b) *Samples with known intentional addition of milk.*

The following parameters were determined within the interlaboratory study: repeatability, reproducibility, LOD, LOQ, ratio of false-negative (FN/TP), and false-positive results (FP/TN), respectively.

(1) *LOD and LOQ.*—LOQ (sometimes called lowest observable quantity) of the test kit was judged as 10 times the value of an average SD of reproducibility ($LOQ = 10 \cdot s_R$) on levels close to LOQ. A value of 1.8 mg/kg was found. LOD of the kit was judged as three times the value of an average SD of reproducibility ($LOD = 3 \cdot s_R$). A value of 0.5 mg/kg was determined. LOQ is slightly higher than the lowest point in the calibration curve (1.0 mg CAS/kg) and reflects concentrations above which the presence of casein is beyond reasonable doubt, at a probability level of 95%.

(2) *False-positive ratio versus false-negative ratio (FP/FN).*—Based on the assumption that samples in

Table 5. Repeatability and reproducibility data from the interlaboratory study

Performance characteristics		Sample No.							
		CS2	2	3	5	6	9	10	12
Mean value, mg/kg	y	4.47	15.66	4.15	13.19	4.75	17.72	9.26	15.01
Number of laboratories (after exclusion of outliers)	n	7	8	7	8	8	8	8	7
Number of outliers	n	0	0	1	0	0	0	0	0
Repeatability SD	s _r	0.52	15.48	2.13	6.18	2.48	2.60	1.35	3.01
RSD of repeatability, %	RSD _r	11.53	98.84	51.39	46.90	52.17	14.69	14.53	20.03
Repeatability limit	r	1.44	43.33	5.97	17.32	6.93	7.29	3.77	8.42
Reproducibility SD	s _R	0.60	17.36	3.97	8.03	2.84	5.75	3.32	6.85
RSD of reproducibility, %	RSD _R	13.35	110.88	95.63	60.92	59.80	32.48	35.89	45.60
Reproducibility limit	R	1.67	48.61	11.11	22.49	7.95	16.11	9.31	19.17
r/R ratio	r/R	0.86	0.89	0.54	0.77	0.87	0.45	0.40	0.44
RSD of reproducibility from Horwitz equation, %	CV(R)	12.77	10.58	12.92	10.85	12.66	10.38	11.44	10.64
Recovery	%	89.4	78.3	83.0	65.9	94.9	—	—	—

category 1a above are truly negative, one can count the number of false statements, i.e., statements that claim the negative samples to be positive samples and are therefore FP statements. As a decision point for categorization into the FP category, the findings can be either higher than the lowest calibration point (i.e., 1 mg/kg) or higher than LOQ (1.8 mg/kg). Similarly, in category 2, the number of FN statements can be determined as the number of false statements, i.e., those when a positive result was claimed as negative. An overview of results of the above-mentioned statements FP and FN are presented in Table 4. TP = True positive; TN = true negative; FN = false-negative; FP = false-positive; F/T = ratio of number of false statements to number of true statements.

As presented in Table 4, no FN samples were found, while the ratio FP/TN was 0.11 (i.e., 11%) when 1.8 mg CAS/kg was used as a decision limit. Presented F/T ratios can be generally considered low and acceptable for the intended use of the kit. These ratios characterize the reliability and selectivity of the test kit. All three FP results were very close to the lowest calibration level and could be caused by cross-contamination of samples and therefore regarded as laboratory fault.

(3) *Repeatability and reproducibility.*—Values of repeatability and reproducibility were calculated from eight samples that were suitable for quantification, i.e., samples from category 2. Results are summarized in Table 5. While repeatability (r) and reproducibility (R) expressed as RSDs of control and naturally contaminated samples (9, 10, 12) have acceptable (RSD_r from 11 to 20% and RSD_R from 13 to 46%), the same characteristics (r and R) in spiked samples (2, 3, 5, 6) are significantly higher (RSD_r from 47 to 98% and RSD_R from 60 to 111%). These differences are probably caused by

complications in the fortification procedure and could be related to inhomogeneity of the spiked samples. Although the values of the RSD_R, % in naturally contaminated samples as well as in the control samples are slightly higher than could be expected from the Horwitz equation (20; see Table 5, line Horwitz CV_R in %), they can still be generally considered as acceptable performance characteristics for intended use, i.e., for reliable proof of presence of casein and its quantification.

Cochran's and Grubbs' tests according to ISO 5725 were used for detection of outliers (21). Probability level = 1% was used for exclusion of laboratory from further statistical evaluation.

(4) *Trueness.*—Five samples with known concentrations of casein at two different levels (5 and 20 mg/kg) were included in the determination of recovery. The recoveries obtained are shown in Table 5. Recoveries lie between 66 and 94%, whereas the average recovery was 82%. In spite of the large repeatability and reproducibility for these samples, the recovery values reached in the study were acceptable and suitable for the intended use of the test kit.

Conclusions

A newly developed competitive ELISA kit for the quantification of casein was validated. Analytical sensitivity, functional sensitivity, recovery, accuracy, and other parameters were determined in an in-house validation.

The interlaboratory study confirmed that the developed ELISA kit is suitable for casein quantification in food products and raw material. All negative samples were determined as negative (less than the LOQ estimated in the study) by all laboratories, and all positive samples were determined as positive by all laboratories as well. LOD and

LOQ were determined within the interlaboratory study. All spiked samples and control sample (CS2) had a casein content higher than the lowest standard (for spiked samples a concentration of 5 mg/kg) and higher than the third or the highest standard (for spiked samples a concentration of 20 mg/kg). Performance characteristics (r, R, recovery) determined in the study were acceptable for routine use of the casein test kit in food control laboratories.

Tracing of allergens in food has recently become quite important as food allergens have been recognized as the cause of many symptoms of atopy in predisposed consumers. Some commercial kits for detection of food allergens are already available. However, the spectrum of food allergens is not covered in full; confirmation of detected quantities is possible only with systems based on more than one kit. Also, in some foods, a number of substances can compose its allergenicity. Therefore, it is an advantage to detect more than one allergen present in a particular food. The ELISA kit used in this study fulfills the need for a tool to detect casein, a cow's milk allergen. Validation of the kit in an interlaboratory study means that it was tested on a broad scale of food matrixes, which increases its value as a versatile detection and quantification tool.

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