

Determination of Cry9C Protein in Corn-Based Foods by Enzyme-Linked Immunosorbent Assay: Interlaboratory Study

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The performance of a commercially available enzyme-linked immunosorbent assay kit (EnviroLogix) was assessed for the determination of Cry9C protein, which is produced by the genetically modified corn StarLink, in 8 types of corn-based foods (starch, refined oil, soft tortillas, tortilla chips, corn flakes, corn puffs, corn muffins, and corn bread) in an interlaboratory study involving 7 laboratories in the United States. The assay kit is a double antibody sandwich and is based on the specific interaction between antibody and antigen. The Cry9C protein analyte is sandwiched between 2 antibodies, one to capture the analyte and the other is conjugated to the enzyme, horseradish peroxidase. The enzyme uses tetramethylbenzidine/peroxide for color development. A strong acid stopping reagent is then used to change the color from blue to a stable yellow. The intensity of the color is proportional to the concentration of the Cry9C protein. In this study blind duplicates of control samples (blank material prepared from non-StarLink corn), spiked samples (blank material with the addition of Cry9C protein), and samples containing incurred analyte (products prepared with StarLink corn) were analyzed. Cry9C protein from 2 different sources was used to spike the food products. Cry9C protein produced and purified from a bacterial host was used to prepare spiked test samples at 2.72 and 6.8 ng/g. Cry9C protein from StarLink corn flour was used to prepare spiked samples at 1.97 ng/g. Average recoveries for samples spiked with corn flour Cry9C protein at 1.97 ng/g ranged from 73 to 122%, within-laboratory relative standard deviations (RSD_w) ranged from 6 to 22%, and between-laboratories relative standard deviations (RSD_b) ranged from 16 to 56%. Average recoveries for samples spiked with bacterial Cry9C protein at 2.72 and 6.8 ng/g ranged from 27 to 96% and from 32 to 113%, respectively; RSD_w values ranged from 10 to 35% and from 7 to 38%, respectively; and the RSD_b ranged from 28 to 84% and 15 to 75%, respec-

tively. The incurred test samples were found to contain Cry9C protein at levels ranging from 0.8 to 3187 ng/g depending on the product, RSD_w values ranged from 5 to 16% and RSD_b values ranged from 11 to 71%. Results of the statistical analysis indicate that this method is applicable to the determination of Cry9C protein in the 8 types of collaboratively studied corn-based products containing Cry9C protein (from StarLink) at levels of ≥ 2 ng/g.

StarLink, a genetically modified corn that produces an insecticidal toxin, i.e., Cry9C protein derived from *Bacillus thuringiensis* (Bt), was approved by the U.S. Environmental Protection Agency in 1998 for use as animal feed (1). Approval of StarLink corn for human use was withheld on the grounds that the Cry9C protein might pose a risk of allergic reactions in humans. The possibility of human allergic reaction to Cry9C protein has not been demonstrated.

In September 2000, trace amounts of StarLink DNA were found in corn-based taco shells, resulting in a national and international recall of the taco shells. The method used for the detection of StarLink DNA was based on the polymerase chain reaction (PCR); however, this method is very time consuming and requires a high level of technical expertise and the use of specific primers. Another analytical approach for the detection of StarLink corn is to analyze for the Cry9C protein. Currently, 4 manufacturers, Agdia, (Elkhart, IN), Strategic Diagnostics, Inc. (Newark, DE), Neogen Corp. (Lansing, MI), and EnviroLogix, Inc. (Portland, ME), produce enzyme-linked immunosorbent assay (ELISA) test kits for the detection of Cry9C protein in corn, cornmeal, and grits. The results obtained with the first 3 kits are expressed as the percentage of StarLink corn contamination present in the test matrix. The claimed limit of determination of these kits ranges from 0.01 to 0.125% of StarLink corn. The results obtained with the EnviroLogix kit by using a polyclonal antibody-based technique are expressed as ng protein/g corn product with a claimed limit of determination of approximately 1 ng/g. Polyclonal antibodies recognize multiple epitopes on proteins. Some epitopes may remain after denaturation and fragmentation of the native protein whereas others may be destroyed. The ability of the ELISA to detect the Cry9C protein could be affected by processing which could po-

Table 1. Interlaboratory study results for the determination of Cry9C protein in starch, oil, soft tortillas, tortilla chips, corn puffs, corn flakes, corn muffins, and corn bread

Matrix	Level present, ng/g	No. of labs ^a	Mean found, ng/g	Rec., %	S _r , ng/g	RSD _r , %	S _R , ng/g	RSD _R , %	r	R	HORRAT
Starch	<0.5 ^b	6(0)	ND ^c								
	2.72 ^d	6(0)	0.91	33.5	0.32	35.0	0.70	77.3	0.90	2.0	2.0
	6.80 ^d	5(1)	2.63	38.7	0.96	12.2	0.82	31.2	2.72	2.32	0.8
	1.97 ^e	6(0)	1.89	95.9	0.27	14.0	0.48	25.2	0.75	1.35	0.6
	Incurred ^f	6(0)	27.60		2.61	9.5	3.10	11.2	7.37	8.75	0.4
Oil	<0.5 ^b	6(0)	ND								
	2.72 ^d	6(0)	0.73	26.9	0.13	12.9	0.62	84.8	0.37	1.76	2.2
	6.80 ^d	5(1)	2.15	32.2	0.22	10.4	1.62	75.4	0.63	4.59	2.4
	1.97 ^e	6(0)	1.69	85.3	0.11	6.4	0.45	26.7	0.31	1.27	0.7
	Incurred ^f	5(1)	18.60		3.27	5.9	6.02	21.2	9.24	17	1.0
Tortillas soft ^g	<0.5 ^b	6(0)	ND								
	2.72 ^d	6(0)	1.68	61.6			0.97	58.0		2.72	1.5
	6.80 ^d	6(0)	4.04	59.4	0.74	18.3	2.22	54.9	2.09	6.28	1.6
	1.97 ^e	6(0)	1.70	86.0			0.37	21.7		1.04	0.5
	Incurred ^f	6(0)	22.7		2.03	8.9	2.02	8.9	5.7	13.5	0.8
Tortilla chips ^g	<0.5 ^b	6(0)	ND								
	2.72 ^d	6(0)	2.48	91.2			0.87	35.0		2.44	1.0
	6.80 ^d	6(0)	6.49	95.3	0.82	12.6	1.63	25.1	2.31	4.60	0.8
	1.97 ^e	6(0)	2.04	103.6			0.40	19.6		1.12	0.5
	Incurred ^f	6(0)	17.9		0.82	4.7	3.26	18.9	2.32	9.22	0.6
Corn puffs	<0.5 ^b	6(0)	0								
	2.72 ^d	6(0)	1.23	45.2	0.34	27.7	1.01	82.5	0.96	2.87	2.1
	6.80 ^d	6(0)	3.53	51.9	1.35	38.3	2.59	73.4	3.82	7.32	2.2
	1.97 ^e	6(0)	1.44	73.1	0.32	22.3	0.80	55.6	0.91	2.27	1.4
	Incurred ^f	5(1)	7.34		1.13	15.4	2.42	32.9	3.19	6.83	1
Corn flakes	<0.5 ^b	6(0)	0								
	2.72 ^d	6(0)	2.62	96.3	0.26	9.8	1.20	45.8	0.73	3.40	1.2
	6.80 ^d	6(0)	7.68	112.9	0.57	7.4	1.93	25.2	1.6	5.47	0.7
	1.97 ^e	6(0)	2.4	121.8	0.18	7.53	0.49	20.2	0.51	1.37	0.5
	Incurred ^f	6(0)	0.78		0.3	41.7	0.56	71.3	0.93	1.59	1.4
Corn muffins	<0.5 ^b	6(0)	0								
	2.72 ^d	6(0)	1.62	59.6	0.23	14.4	0.67	41.5	0.66	1.9	1.1
	6.80 ^d	6(0)	5.26	77.4	0.83	15.8	0.76	14.5	2.34	2.16	0.4
	1.97 ^e	5(1)	1.64	85.4	0.17	10.4	0.37	22.3	0.49	1.04	0.6
	Incurred ^f	6(0)	767.1		110	14.3	161.3	21.0	310.0	456.3	1.3
Corn bread	<0.5 ^b	6(0)	0								
	2.72 ^d	6(0)	1.72	63.2	0.16	9.5	0.48	27.8	0.46	1.35	0.7
	6.80 ^d	6(0)	5.08	74.7	0.88	17.3	0.81	16.0	2.49	2.3	0.5
	1.97 ^e	6(0)	1.6	81.2	0.12	7.2	0.26	16.4	0.33	0.74	0.4
	Incurred ^f	6(0)	3187		154	4.8	854	26.8	434	2416	2

^a Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

^b Blank sample (prepared with non-StarLink corn).

^c ND = not detected (<0.5 ng/g); results between 0.0 and 0.44 ng/g were rounded down to 0 ng/g.

^d Sample spiked with bacterial Cry9C protein.

^e Sample spiked with Cry9C protein from StarLink corn flour.

^f Sample prepared with StarLink corn.

^g No blind duplicates of soft tortillas and tortilla chips at 2.72 and 1.97 ng/g were prepared because of the limited non-Cry9C materials.

Table 2. Interlaboratory study results (no data were removed) for the determination of Cry9C protein in corn-based foods

Food	Recovery, %	Mean, ng/g	RSD _r , %	RSD _R , %
Spiking level, 2.72 ng/g ^a				
Starch	32	0.88	32	36
Oil	27	0.73	18	85
Soft tortillas ^b	61	1.67		58
Tortilla chips ^b	91	2.48		35
Corn puffs	43	1.17	39	92
Corn flakes	96	2.62	11	46
Corn muffins	60	1.62	14	41
Corn bread	63	1.72	9	28
Spiking level, 6.8 ng/g ^a				
Starch	41	2.76	32	35
Oil	28	1.93	26	82
Soft tortillas ^b	59	4.04	18	55
Tortilla chips ^b	95	6.49	13	25
Corn puffs	52	3.53	38	73
Corn flakes	113	7.68	7	25
Corn muffins	77	5.26	16	15
Corn bread	75	5.08	17	17
Spiking level, 1.97 ng/g ^c				
Starch	96	1.89	14	25
Oil	85	1.68	6	27
Soft tortillas ^b	87	1.71		28
Tortilla chips ^b	104	2.04		20
Corn puffs	73	1.44	22	56
Corn flakes	122	2.40	8	20
Corn muffins	79	1.56	25	30
Corn bread	81	1.60	7	16
Level incurred, ng/g ^d				
Starch		27.6	10	11
Oil		17.2	16	29
Soft tortillas ^b		20.9	9	23
Tortilla chips ^b		17.9	5	15
Corn puffs		6.7	16	41
Corn flakes		0.8	51	80
Corn muffins		767	14	21
Corn bread		3180	5	26

^a Food was spiked with bacterial Cry9C protein.^b Single analysis.^c Food was spiked with protein from StarLink corn flour.^d Food was prepared with StarLink corn.

tentially alter the nature of the Cry9C protein, resulting in the loss of some epitopes. Therefore, the EnviroLogix polyclonal antibody-based ELISA kit may be underestimating the level of the Cry9C protein, especially in highly processed food products. Our study was not designed to validate the applicability of the antibodies used in the ELISA to detect all forms of the Cry9C protein.

The purpose of this interlaboratory study was to determine the accuracy, repeatability, and reproducibility parameters of the EnviroLogix ELISA kit method for the determination of Cry9C protein in processed foods. In this study, the matrixes included representative corn-based food products used in the United States, including starch and oil; minimally processed foods such as muffins and bread; and more highly processed foods such as corn puffs and corn flakes breakfast cereals, soft tortillas, and tortilla chips. Duplicates of a non-StarLink corn flour test sample were also included in this study as controls.

Interlaboratory Study

All corn-based food products, Cry9C protein, StarLink corn flour, and non-StarLink corn flour were provided by Aventis CropScience (Research Triangle Park, NC).

Preparation of Test Materials

Starch and refined oil (not bleached or deodorized) were wet-milled commodities. The processed foods were prepared in laboratories by using pilot-scale procedures. Soft tortillas and tortilla chips were prepared by cold-pressing masa; soft tortillas, by baking in an oven; tortilla chips, by frying in canola oil; corn puffs, by an extrusion process with no additives; and corn muffins and corn bread, by baking in an oven. For each matrix (5 sets/matrix), duplicate test portions were prepared including a blank material (prepared with non-StarLink corn), 3 spiked materials (to be spiked by the participants), a material containing incurred analyte (prepared with StarLink corn), and a non-StarLink corn flour. The test materials, except the oil, were mixed with powdered dry ice and homogenized at high speed in a stainless steel blender jar. After evaporation of the dry ice, 1 ± 0.01 g test portions were weighed into 50 mL polypropylene centrifuge tubes.

Preparation of Spiking Solutions

Spiking solutions A and B were prepared from Cry9C protein that was produced and purified from a bacterial host. A stock solution of the Cry9C protein at 136 ng/mL was prepared in phosphate-buffered saline with bovine serum albumin added as the supporting protein. Spiking solution A containing 68 ng Cry9C protein/mL and spiking solution B containing 27.2 ng Cry9C protein/mL were prepared by diluting the stock solution 1:2 and 1:5, respectively. Aliquots of the spiking solutions were pipetted into 1.5 mL Eppendorf tubes (1 tube spiking solution for each test portion), and each tube was labeled with the corresponding random number of the test sample.

Spiking solution C was prepared by extracting a mixture of 39 mg 100% StarLink corn flour and 39 g non-StarLink corn flour with 195 mL 1X grain extraction buffer (a 20X grain ex-

traction buffer is included in the ELISA kit) to give 3.94 ng Cry9C protein/mL, or 0.2 mg StarLink corn flour/mL solution (the Cry9C protein level in corn flour was 19.7 µg/g). This solution contained both Cry9C protein and other proteins extracted from the corn flour. Aliquots of the spiking solution were pipetted into 1.5 mL Eppendorf tubes (1 tube spiking solution for each test portion, and each tube was labeled with the corresponding random number of the test sample).

Preparation of Positive Controls

Positive control A, containing bacterial host Cry9C protein at 1.36 ng/mL, was prepared by diluting (1:100) the stock solution containing 136 ng protein/mL (see *Preparation of Spiking Solutions* above). Positive control B, containing Cry9C protein from StarLink corn at 0.394 ng/mL, was prepared by diluting (1:10) spiking solution C (see *Preparation of Spiking Solutions* above) with non-StarLink corn extract (see *Preparation of Negative Control* below).

Preparation of Negative Control

The negative control (0.1 g non-StarLink corn flour/mL) was prepared by extracting 15 g non-StarLink corn flour with 150 mL 1X grain extraction buffer.

Preparation of Practice Samples

Six practice samples of non-StarLink corn-based foods (cornmeal, corn flakes, and corn bread prepared in duplicate) were sent to collaborators. To become familiar with the analytical method before proceeding with the interlaboratory study, the participants were instructed to spike the samples at 6.8 ng protein/g, by using the labeled spiking solution A before analysis. All test materials and solutions were kept frozen and packed in dry ice before they were shipped to collaborators.

The study was designed and conducted in accordance with the guidelines prescribed by AOAC INTERNATIONAL (2). Only 7 collaborators were included in the study because of the limited test materials available. The 7 laboratories represented the U.S. government, academia, and industry. These laboratories were selected on the basis of their demonstrated experience with immunoassays; however, 4 of the laboratories had no prior experience in using the EnviroLogix kits. Each laboratory was assigned a code number.

Each participant was supplied with the test materials (78 test samples), 44 spiking solutions, 2 positive controls, 1 negative control, and 6 practice samples. The collaborators were provided a packing list, instructions to collaborators, test procedure, ELISA template forms, and report forms for results and general comments.

Participants were requested to analyze 6 practice test samples spiked at 6.8 ng/g before proceeding with the interlaboratory study; they were not to proceed with the interlaboratory study unless recoveries were within 70–135% of the spiked level. Participants obtaining results out of this range were instructed to contact the Study Director before proceeding with the analyses.

The collaborators were given a set of instructions:

Table 3. Interlaboratory study results (outlier data were removed) for the determination of Cry9C protein in corn-based foods

Food	Recovery, %	Mean, ng/g	RSD _r , %	RSD _R , %
Spiking level, 2.72 ng/g ^a				
Starch	33.5	0.91	35	77
Oil	26.9	0.73	18	84
Soft tortillas ^b	61.6	1.68		58
Tortilla chips ^b	91.2	2.48		35
Corn puffs	45.2	1.23	28	83
Corn flakes	96.3	2.62	10	46
Corn muffins	59.6	1.62	14	42
Corn bread	63.2	1.72	10	28
Spiking level, 6.8 ng/g ^a				
Starch	38.7	2.63	12	31
Oil	32.2	2.15	10	75
Soft tortillas ^b	59.4	4.04	18	55
Tortilla chips ^b	95.4	6.49	13	25
Corn puffs	51.9	3.52	38	73
Corn flakes	112.9	7.68	7	25
Corn muffins	77.4	5.26	16	15
Corn bread	64.7	5.08	17	16
Spiking level, 1.97 ng/g ^c				
Starch	95.9	1.89	14	25
Oil	85.5	1.69	6	27
Soft tortillas ^b	86.0	1.7		22
Tortilla chips ^b	103.6	2.04		20
Corn puffs	73.1	1.44	22	56
Corn flakes	121.8	2.4	8	20
Corn muffins	78.2	1.64	10	22
Corn bread	81.2	1.6	7	16
Level incurred, ng/g ^d				
Starch		27.6	10	11
Oil		18.6	6	21
Soft tortillas ^b		22.7	9	9
Tortilla chips ^b		17.9	5	19
Corn puffs		7.3	15	33
Corn flakes		0.8	42	71
Corn muffins		767	14	21
Corn bread		3187	5	27

^a Food was spiked with bacterial Cry9C protein.

^b Single analysis.

^c Food was spiked with protein from StarLink corn flour.

^d Food was prepared with StarLink corn.

Table 4. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate starch samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	ND	0.91	1.25	1.70	ND	0.90
	ND	1.22	1.32	1.40	ND	1.90
6.80 ^c	2.39	2.52	3.06	3.60	1.42	1.94 ^d
	3.24	2.45	2.52	3.60	1.52	4.81 ^d
1.97 ^e	1.26	1.65	1.98	2.30	1.42	2.54
	1.51	2.33	1.89	2.50	1.30	2.03
Incurred ^f	24.6	26.7	27.0	24.9	33.0	33.8
	27.9	27.3	27.0	23.6	26.8	28.3

^a Blank sample (prepared with non-StarLink corn).

^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.

^c Sample spiked with bacterial Cry9C protein.

^d Data rejected on the basis of the Cochran test.

^e Sample spiked with Cry9C protein from StarLink corn flour.

^f Sample prepared with StarLink corn.

(1) *Practice samples*.—Add 100 µL spiking solution A (68 ng/mL solution) to each test portion (1 g), mix on vortex mixer, then add 9.90 mL extraction solvent, and follow method procedure.

(2) *Four sets of test portions*.—Match code number of each spiking solution with code number of each test portion (1 g). Add 100 µL spiking solution, mix, add 9.90 mL extraction solvent, mix on vortex mixer for 1 min, and perform analysis (the solutions of unknown concentration were portions of spiking solutions A and B).

(3) *Two sets of test portions*.—Match code number of each spiking solution with code number of each test portion (1 g), add 500 µL spiking solution, mix, add 9.50 µL extraction solution (the solutions of unknown concentration were portions of spiking solution C).

(4) Extract and assay a maximum of 16 test portions on the same day.

Caution: Cry9C protein is currently considered a potential food allergen. Wear gloves and safety glasses at all times. Conduct all standard and sample preparation stages in a fume hood.

METHOD

See Table 1 for the results of the interlaboratory study supporting acceptance of the method.

Table 5. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate oil samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	ND	1.00	0.85	1.20	ND	1.27
	ND	0.84	0.74	1.20	ND	1.68
6.80 ^c	ND ^d	1.53	2.43	2.60	ND	4.59
	1.60 ^d	1.22	2.14	2.90	ND	4.11
1.97 ^e	0.94	1.74	1.63	2.20	1.57	2.01
	0.99	2.01	1.45	2.30	1.50	1.88
Incurred ^f	25.05	13.96	18.96	17.67	ND ^d	33.58
	21.97	14.75	18.56	17.92	ND ^d	23.76

^a Blank sample (prepared with non-StarLink corn).

^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.

^c Sample spiked with bacterial Cry9C protein.

^d Data rejected on the basis of the Cochran test.

^e Sample spiked with Cry9C protein from StarLink corn flour.

^f Sample prepared with StarLink corn.

Principle

Test corn product is extracted with extraction buffer. The extract is added to test wells coated with polyclonal antibodies raised against Cry9C protein. The Cry9C protein in the extract is sandwiched between 2 antibodies, one to capture the analyte and the other conjugated to the enzyme, horseradish peroxidase. Tetramethylbenzidine/peroxide substrate is added to produce a blue color. The reaction is stopped with a stopping solution, and the color changes from blue to yellow. The color, which is proportional to the concentration of the Cry9C protein, is read by using an enzyme-linked immunosorbent assay (ELISA) reader. A linear plot of absorbance versus concentration of the standards is used to quantitate Cry9C protein in the test extract.

Apparatus

(a) *Centrifuge*.—Operating at >23 500 × *g*.

(b) *Shaker*.—IKA-Schuttler MTS 4 shaker (Fisher Scientific, Pittsburgh, PA, orbital shaker, or equivalent).

(c) *Pipets and pipet tips*.—20, 100, and 1000 µL, and 10 mL adjustable air displacement pipets with disposable tips.

(d) *Multichannel pipet*.—Labsystems 8- or 12-channel, 200 µL (Fisher Scientific, or equivalent).

(e) *ELISA reader*.—Microwell spectrophotometer with variable wavelength, Spectra Max 340 (Molecular Devices Corp., Sunnyvale, CA, or equivalent).

(f) *Microwell holder*.—Dynatech 96-well holder (Fisher Scientific).

Table 6. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate soft tortillas samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	1.11 ^d	1.83 ^d	2.17 ^d	2.30 ^d	ND ^d	2.64 ^d
6.80 ^c	4.42	1.83	5.21	5.90	0.52	6.83
	5.36	2.36	4.87	5.90	0.75	4.54
1.97 ^e	1.42 ^d	1.87 ^d	1.72 ^d	2.10 ^d	1.11 ^d	1.95 ^d
Incurred ^f	23.58	21.33	24.69	24.90	11.74 ^g	24.46
	22.64	20.18	19.38	21.60	11.81 ^g	24.64

^a Blank sample (prepared with non-StarLink corn).^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.^c Sample spiked with bacterial Cry9C protein.^d No blind duplicates of soft tortillas at 2.72 and 1.97 ng/g were prepared because of the shortage of non-Cry9C materials.^e Sample spiked with Cry9C protein from StarLink corn flour.^f Sample prepared with StarLink corn.^g Data rejected on the basis of the Grubbs test.

(g) *Reagent boats*.—Plastic 30 mL wedge-shaped troughs for use with multichannel pipet.

(h) *Acetate plate sealers*.

(i) *Eppendorf tubes*.

(j) *Microdilution tubes*.—1.2 mL polypropylene, 96 in a rack, similar to a 96-well ELISA plate (Fisher Scientific). Do not use glass tubes.

(k) *Centrifuge tube*.—50 mL, polypropylene.

Reagents

(Store all reagents except working extraction buffer at 4°C. Allow all reagents to reach ambient temperature before use.)

Reagents (a)–(g) are commercially available in the Cry9C ELISA Plate Kit (Cat. No. AP 008, EnviroLogix, Inc., 55 Industrial Way, Portland, ME 04103, USA; +1-207-797-0300).

(a) *Anti-Cry9C protein antibody-enzyme conjugate*.—Liquid horseradish peroxidase conjugated anti-Cry9C protein antibody (11.5 mL). Store at 4°C.

(b) *Antibody-coated solid support*.—12 strips of 8 antibody-coated wells, in plate frame.

(c) *Enzyme substrate*.—Tetramethylbenzidine/peroxide (11.5 mL).

(d) *Stopping solution*.—1.0M HCl.

(e) *Standard solutions*.—0, 0.2, 1.0, and 2.5 ng/mL. (When a 1 g test sample is extracted with 10 mL extraction solvent, a dilution factor of 10 is used. The standard solutions are then equivalent to 0, 2, 10, and 25 ng of Cry9C protein/g food.)

Table 7. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate tortilla chip samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	2.14 ^d	2.86 ^d	3.02 ^d	2.60 ^d	0.92 ^d	3.37 ^d
6.80 ^c	5.92	7.13	8.01	6.40	3.32	8.84
	6.72	6.19	7.66	7.20	3.95	6.54
1.97 ^e	1.69 ^d	2.03 ^d	2.23 ^d	2.70 ^d	1.59 ^d	2.00 ^d
Incurred ^f	19.56	18.85	19.95	17.60	13.58	19.07
	20.47	16.41	20.47	16.50	13.21	19.42

^a Blank sample (prepared with non-StarLink corn).^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.^c Sample spiked with bacterial Cry9C protein.^d No blind duplicates of soft tortillas at 2.72 and 1.97 ng/g were prepared because of the shortage of non-Cry9C materials.^e Sample spiked with Cry9C protein from StarLink corn flour.^f Sample prepared with StarLink corn.

(f) *Extraction buffer*.—Prepare 1X grain extraction buffer by mixing 5 mL 20X grain extraction buffer with 95 mL distilled water. Store at room temperature.

(g) *Washing buffer*.—Add contents of packet of wash buffer (phosphate-buffered saline/0.05% Tween 20; Cat. No. P-3563, Sigma, St. Louis, MO) to 1 L distilled water, and stir to dissolve.

Extraction

Add 10 mL 1X extraction buffer to 50 mL polypropylene centrifuge tube containing 1 g test sample. Cap tube and shake tube vigorously by hand, or mix with vortex mixer for 1 min. Place tubes horizontally on shaker, set at 700 rpm, and shake tube for 2 h at room temperature. Centrifuge at 23 500 × g, or use Sorvall GSA rotor at 12 000 rpm (or equivalent) for 10 min at 2–4°C.

Prepare template of analysis on ELISA sheet. List standard solutions, test extracts, positive controls, negative controls, and 1X grain extraction solution in duplicate. Use polypropylene transfer pipet to transfer 2 aliquots (approximately 0.4 mL each) of standard solutions; 0, 0.2, 1.0, and 2.5 ng/mL, into separate 1.2 microdilution tubes (96 tubes in rack, similar to ELISA well plate) or polypropylene ELISA wells.

Use polypropylene transfer pipet to transfer 2 aliquots (approximately 0.4 mL each) of each supernatant (place pipet in clear part of supernatant) into separate 1.2 mL microdilution tubes (96 tubes in rack, similar to ELISA well plate). Number microdilution tubes. Use polypropylene transfer pipet to transfer 2 aliquots (approximately 0.2 mL each) of positive

Table 8. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate corn puffs samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	ND	ND	2.51	1.90	1.44	ND
	ND	1.36	2.47	2.50	1.34	0.50
6.80 ^c	ND	1.55	2.81	5.10	5.58	4.41
	ND	0.77	5.71	3.50	5.28	7.60
1.97 ^d	ND	1.52	1.40	2.20	1.48	2.18
	ND	2.33	1.23	2.10	1.44	1.44
Incurred ^e	6.92	3.67	9.27	9.00	2.78 ^f	5.77
	7.06	3.69	9.56	9.10	3.86 ^f	9.32

^a Blank sample (prepared with non-StarLink corn).^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.^c Sample spiked with bacterial Cry9C protein.^d Sample spiked with Cry9C protein from StarLink corn flour.^e Sample prepared with StarLink corn.^f Data rejected on the basis of the Cochran test.**Table 9. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate corn flakes samples**

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	2.13	2.69	3.53	3.40	ND	3.69
	2.00	2.96	3.51	3.20	0.86	3.40
6.80 ^c	6.44	7.07	9.03	8.40	5.46	10.49
	7.65	6.00	9.01	8.00	4.50	10.10
1.97 ^d	2.29	2.70	2.62	2.70	1.55	2.32
	1.96	3.11	2.68	2.90	1.80	2.22
Incurred ^e	0.50	1.67	0.50	1.70	0.53	ND
	ND	1.57	0.50	0.70	0.66	0.73

^a Blank sample (prepared with non-StarLink corn).^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.^c Sample spiked with bacterial Cry9C protein.^d Sample spiked with Cry9C protein from StarLink corn flour.^e Sample prepared with StarLink corn.

controls and negative control into separate 1.2 microdilution tubes (96 tubes in rack, similar to ELISA well plate). Save extracts in refrigerator. Repeat ELISA next day if further dilution of test extracts is necessary.

Conducting the Test

Mark each strip of wells with a number and place strips in well holder with all marked ends on same side. Prepare ELISA template. (Make sure the order of the template is the same as that of the microdilution tubes.) Mix each reagent by swirling reagent bottle before use. Using multichannel pipet, transfer duplicate standard solutions, duplicate test extracts, duplicate positive controls, duplicate negative controls, and duplicate 1X grain extraction solutions from microdilution tubes to antibody-coated wells. Cover wells with plate sealer. Place microwell holder on shaker set at 200 rpm for 30 min. Cover microdilution tubes. Place tubes and rest of supernatant (in 50 mL centrifuge tubes) in refrigerator and save in case repeat of ELISA analysis is needed.

After shaking microwell holder, carefully remove covering, and vigorously shake contents of wells into a sink or other suitable container. Flood wells completely with wash buffer, and then shake to empty wells. Repeat this wash step 3 times. Finally, tap plate on paper towel until all remaining water has been removed.

Measure 5.7 mL anti-Cry9C protein antibody-enzyme conjugate (for 6 strips) into reagent boat, and use multichannel pipet to add 100 μ L to each well. Cover wells with new plate

sealer to prevent evaporation, and incubate at ambient temperature for 2 h on orbital plate shaker at 200 rpm.

After incubation, carefully remove cover and vigorously shake contents of wells into sink or other suitable container. Flood wells completely with wash buffer, and shake to empty wells. Repeat this wash step 3 times. Tap plate on paper towel until all remaining water has been removed.

Measure 5.7 mL substrate (for 6 strips of wells, 8 wells/strip) into reagent boat, using multichannel pipet to add 100 μ L to each well. Cover wells with new plate sealer, and incubate for 30 min at ambient temperature in the dark on orbital shaker at 200 rpm.

Measure 5.7 mL stop solution (for 6 strips) into reagent boat, using multichannel pipet to add 100 μ L to each well. This will turn the well contents yellow. Place plate on orbital shaker, and shake for 30 s at 200 rpm. Read plate immediately (never >5 min after addition of stop solution).

Spectrophotometric Measurement

Set wavelength of microtiter plate reader at 450 nm. (If dual wavelength capability is available, use 650 nm as reference wavelength.) Set plate reader blank on 0 standard wells (0 ng/g standard); this should automatically subtract the mean absorbance (A) from each remaining calibration standard and test (A). If reader cannot do this, it must be done manually.

Use linear fit for standard curve if microtiter plate reader has data reduction capabilities. If not, calculate results manually.

Table 10. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate corn muffin samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	1.23	1.84	2.09	1.9	0.73	2.33
	0.55	2.00	2.06	2.1	0.65	1.98
6.80 ^c	4.17	5.33	5.84	5.1	5.45	6.75
	4.87	4.41	5.85	5.4	5.74	4.16
1.97 ^d	1.22	1.69	1.60	1.9	1.71	0.53 ^e
	1.03	2.13	1.46	2.1	1.63	1.76 ^e
Incurred ^f	607.2	844.2	568.7	970.2	828	1069
	622.0	773.1	671.4	732.8	629.5	889

^a Blank sample (prepared with non-StarLink corn).
^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.
^c Sample spiked with bacterial Cry9C protein.
^d Sample spiked with Cry9C protein from StarLink corn flour.
^e Data rejected on the basis of the Grubbs test.
^f Sample prepared with StarLink corn.

Calculation

Average A of each set of calibration standards and test samples, and subtract average A of 0 ng/g standard from each. Plot mean A of each calibration standard versus corresponding Cry9C concentration on linear scale. Determine Cry9C concentration of each test by finding value of A and corresponding concentration level from graph. Multiply Cry9C protein result in ng/g by 10, the dilution factor (1 g test portion is extracted with 10 mL extraction solvent). If any additional dilutions were performed, multiply by these factors as well.

Precautions/Critical Control Points

- (1) Do not use test kit components beyond expiration date printed on the label (6 months from date of manufacture).
- (2) Do not use reagents from test kits with one serial number with reagents from kits with a different serial number.
- (3) Use of incubation times other than those specified may give inaccurate results.
- (4) Bring all reagents to room temperature (18–30°C) before use by removing reagents from refrigerator and placing at room temperature for 1 h.
- (5) Place unused reagents in refrigerator immediately after use. Avoid prolonged storage of reagents at room temperature.
- (6) Do not freeze test kits.
- (7) Use new pipet tips and plasticware for each test to avoid cross-contamination.

Table 11. Interlaboratory study results as reported by collaborators for the determination of Cry9C protein (ng/g) in blind duplicate corn bread samples

Level, ng/g	Laboratory					
	1	2	4	5	6	7
0 ^a	ND ^b	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND
2.72 ^c	1.08	1.82	2.14	2.00	1.57	1.86
	0.81	1.8	2.23	1.90	1.22	2.18
6.80 ^c	4.29	5.12	5.04	5.30	5.66	5.34
	5.13	4.73	5.96	5.10	3.06	6.24
1.97 ^d	1.28	1.64	1.44	2.00	1.66	1.53
	1.44	1.53	1.32	2.10	1.50	1.80
Incurred ^e	2954	3703	2143	2111	3566	4345
	3028	3559	2608	2134	3766	4337

^a Blank sample (prepared with non-StarLink corn).
^b ND = not detected (<0.5 ng/g); results between 0 and 0.44 ng/g were rounded down to 0 ng/g, and results between 0.45 and 0.49 ng/g were rounded up to 0.5 ng/g.
^c Sample spiked with bacterial Cry9C protein.
^d Sample spiked with Cry9C protein from StarLink corn flour.
^e Sample prepared with StarLink corn.

(8) Prime pipet tips by filling and dispensing once before pipetting each solution.

Results and Discussion

To determine the accuracy of an analytical procedure, it is necessary to have well-characterized spiking solutions. The approximate concentrations of these solutions were determined by the Study Director (*n* = 6), using the same method. For statistical evaluation, the data obtained by the collaborators for the 32 positive control A samples and the 32 positive control B samples were used to calculate the concentrations of the Cry9C spiking solutions and the test samples. For positive control A, no dilution factor was used in the calculations by the collaborators; the number of analyses = 32; the mean = 1.36 ng Cry9C protein/mL; the standard deviation (SD) = 0.13; and the relative standard deviation (RSD) = 9.1%. For positive control B, a factor of 10 was used in the calculations by the collaborators to obtain the protein concentration of spiking solution C because the positive control B was a 1:10 dilution of spiking solution C; the number of analyses = 32; the mean = 3.94 ng Cry9C protein/mL; the SD = 0.52; and the RSD = 13%.

The concentration of the stock bacterial Cry9C protein spiking solution was 136 ng protein/mL. This stock solution was diluted to prepare spiking solutions A and B as indicated in the *Preparation of Spiking Solutions* section. The first 2 spiking levels were 2.72 and 6.8 ng/g (100 µL spiking solu-

Table 12. Cry9C protein and StarLink corn in processed corn-based foods

Foods	Post-processing, ng/g	Pre-processing level, $\mu\text{g/g}^a$	Residual Cry9C, %	StarLink corn in recipe, %	Minimum StarLink corn, % ^b
Starch ^c	27.6	19.7	0.14	100	7.2
Oil ^c	18.6	19.7	0.09	100	10.8
Soft tortillas	22.7	11.4	0.2	58	5.1
Tortilla chips	17.9	14.4	0.14	73	8.2
Corn puffs	7.3	17.7	0.04	90	24.7
Corn flakes	0.8	18.1	NA ^d	92	NA
Corn muffins	767	3.6	21.3	18	0.05
Corn bread	3187	3.6	88.5	18	0.01

^a Calculated value based on the concentration of 19.7 $\mu\text{g/g}$ Cry9C protein detected in StarLink flour as the maximum level of Cry9C detectable in a product made from 100% dry milled StarLink corn.

^b Minimum amounts of StarLink corn in recipes required to quantitate the Cry9C protein in the final corn products.

^c Food processed to remove protein.

^d Not applicable because the level of Cry9C protein is below the limit of quantitation.

tion B with a Cry9C protein concentration of 27.2 ng/mL and 100 μL spiking solution A with a Cry9C protein concentration of 68.0 ng/mL were added to separate 1 g test portions). The concentration of the StarLink corn Cry9C protein spiking solution C was 3.94 ng/mL. The third spiking level was 1.97 ng/g (500 μL spiking solution C was added to a 1 g test portion).

For several reasons the interlaboratory study was limited to 7 collaborators. These included the need for sufficient amounts of the 8 food products prepared with StarLink corn or non-StarLink corn, the handling of a large number of test samples, the participation of available laboratories for such work (large numbers of extractions and analyses), and the limited time for completion of the study. The limited test material was also the reason that no blind duplicates for soft tortillas and tortilla chips spiked at levels of 2.72 and 1.97 ng/g were included in the study. All the participating laboratories completed the interlaboratory study. Problems encountered by 1 of the collaborators during the analysis rendered the data invalid, and the data from that laboratory were not included in the statistical analysis. Recovery data from that laboratory ranged from 0 to 10%, and positive controls were lower than the expected values. The cause of these low recoveries may have been mishandling of the test samples or the ELISA kits during shipment. Data from the remaining 6 laboratories were statistically evaluated for recovery, repeatability, reproducibility, and HORRAT values. The data were tested for outliers by the Cochran, Grubbs, and Paired Grubbs tests. The results from the interlaboratory study for the 8 types of test food samples are presented in Tables 2–11.

Table 2 summarizes the statistical analysis of all data including mean recoveries, within-laboratory relative standard deviations (RSD_i), and between-laboratories relative standard deviations (RSD_R). All control or blank test samples (blank corn-based foods, no addition of Cry9C protein) were found to contain no Cry9C protein; therefore, statistical analysis was not performed for these test samples. In Table 3, the outliers

were excluded, and 0 was substituted for values of <0.44 ng/g. The estimated limit of detection (LOD) was calculated from the results obtained for the duplicate test samples of non-StarLink corn flour included in the study; the estimated LOD was about 0.4–0.5 ng/g ($n = 12$, mean = 0.12, $2\text{SD} = 0.36$). The same data were used to estimate the limit of quantitation (LOQ) for the method. The LOQ was 1.94 ng/g, obtained by using the American Chemical Society definition of the quantitation level (3).

Table 1 summarizes the results from the statistical analysis of the data of the entire study. Data include Cry9C protein mean concentrations, mean recoveries, values for within-laboratory repeatability standard deviation (s_i) and RSD_i , values for between-laboratories reproducibility standard deviation (s_R) and RSD_R , number of sets of acceptable results, number of outlier laboratories, and values for the HORRAT, which has been interpreted as a measure of acceptability of method performance (4). The HORRAT is of very limited value with biochemical reactions probably because of the very low concentrations of the active entities. Determination of outliers was assessed by using the Cochran, Single Grubbs, and Paired Grubbs tests.

Tables 4–11 show the original data of 6 collaborators including data rejected on the basis of the Cochran and Grubbs tests. For statistical analysis, not detected (ND) or 0 has been substituted for data from analyses of spiked test samples showing <0.44 ng/g, because the data were below the estimated LOD.

In general, as shown in Tables 1 and 3, test portions spiked with Cry9C protein produced by bacteria at 2.72 ng protein/g gave lower percent recoveries and higher values of RSD_R than did test portions spiked with Cry9C protein from StarLink corn flour at 1.97 ng protein/g. The bacterial Cry9C protein was used for spiking because it was available in larger amounts and was easier to purify. Cry9C protein from StarLink corn was also used to ensure that the ELISA antibodies could detect plant-produced Cry9C protein in foods.

The RSD_R values for test materials containing incurred analyte were within the acceptable limits.

Performance parameters for the analysis of the 8 corn food products are shown in Table 3. Based on the results for samples (blind duplicates) spiked at 2.72 ng/g (bacterial Cry9C protein), average recoveries ranged from 26.9 to 96.3%, RSD_r values ranged from 10 to 35%, and RSD_R values ranged from 28 to 84%. For the spiking level of 6.8 ng/g (bacterial Cry9C protein), average recoveries ranged from 32 to 113%, RSD_r values ranged from 7 to 38%, and RSD_R values ranged from 15 to 75%. For the spiking level of 1.97 ng/g (StarLink corn protein), average recoveries ranged from 73 to 122%, RSD_r values ranged from 6 to 22%, and RSD_R values ranged from 16 to 56%. The statistics for the test samples containing incurred analyte were comparable to those for samples spiked with StarLink corn protein (RSD_r values ranging from 5 to 42% and RSD_R values ranging from 9 to 71%). The level of Cry9C protein found in the corn flakes test portion containing incurred analyte was 0.8 ng/g and was near the estimated LOD (0.5 ng/g) of the method. This was the lowest level found in a test sample containing incurred analyte. The HORRAT was 1.4. The HORRAT is defined as the ratio of the RSD_R determined in this interlaboratory study to the RSD_R statistically predicted for the determined or known concentration. The predicted RSD_R was calculated by using the following equation:

$$RSD_R = 2^{(1-0.5 \log C)} = 2C^{(-0.1505)}$$

where C = concentration expressed as a decimal fraction (3). A HORRAT of <2 is considered acceptable; the HORRAT values calculated for the present study ranged from 0.4 to 2.4 for test samples spiked with bacterial Cry9C protein. For test samples spiked with StarLink corn protein and test samples of StarLink corn containing incurred analyte, the HORRAT values ranged from 0.4 to 1.4, which were much better than the acceptability threshold value of 2 and much better than values found in most collaborative studies of mycotoxin analytical methods (3, 5) for low ng/g levels.

Table 12 summarizes the effects of processing on the determination of Cry9C protein in corn-based foods. The maximum detectable pre-processing level of Cry9C protein in various foods was estimated based on the level found in corn flour (19.7 µg/g) dry milled from 100% StarLink corn. To calculate the pre-processing value for each of the other foods, the level in flour was multiplied by the percent of StarLink corn in the recipe. The percentage of residual Cry9C protein remaining after processing was calculated from the amount measured by ELISA and the pre-processing estimate (residual Cry9C protein % = post-processing data/pre-processing data × 100). The percentage of StarLink corn in the recipes and the minimum amount of StarLink corn detectable in the finished products are also given (post-processing ng/g: 2 ng/g = StarLink corn in recipe %: minimum StarLink corn %). The LOQ of the method under study is about 2 ng/g.

Minimally processed foods such as muffins and bread exhibited the least degradation of Cry9C protein, thus, requiring

the least amount of StarLink corn in the recipe to obtain a detectable amount of Cry9C protein. In contrast, highly processed foods such as corn puffs exhibited the most degradation in this protein and resulted in larger amounts of StarLink corn required in the recipe to obtain detectable amounts of Cry9C protein. This is also reflected in the residual Cry9C protein values. For example, the residual Cry9C protein in corn bread was about 90% of the protein estimated in the starting materials.

As stated above, the processing of StarLink-containing corn into food products may result in changes in detectability of the protein relative to processing conditions of the food. These may include denaturation, binding to other proteins, conversion to insoluble particles and degradation to peptides and free amino acids. Proteins that are denatured or bound to other proteins may be difficult to dissolve and extract. The extraction method used for the analysis may extract proteins differently from all processed food products. At the present time, there is no reference method to compare the results in the incurred samples. The method studied here may not measure quantitatively all of the Cry9C protein and its degraded products. A more direct approach to address this question would require detailed molecular analysis of the ability of the antibody used in the ELISA to bind Cry9C fragments produced during processing and is beyond the scope of this study.

Collaborators' Comments

All participants indicated that the method was described well and easy to perform. Three participants had no prior experience and 1 participant had limited experience in using the method. Most outliers were confined to the results of 1 laboratory. All participants found it necessary to further dilute the extracts of some of the test samples containing incurred analyte for analysis the next day because the levels of protein were higher than the range of the standard curves.

Conclusions

The observed HORRAT values and the estimated LOQ coupled with the results from the statistical analysis of the data also indicate that the method is applicable the determination of Cry9C protein in 8 types of corn-based products at levels of ≥2 ng/g. Food processing may cause structural changes in Cry9C protein which could alter its antigenic characteristics. Such changes could decrease the amount of Cry9C protein detectable using an antibody-based ELISA assay. This is the first interlaboratory study of a method for the determination of StarLink corn protein in food products. Performance characteristics of this method are similar to those of AOAC INTERNATIONAL Official ELISA Methods for mycotoxins. This abbreviated study was conducted because of an urgent need for a validated method.

Recommendation

Although the study design did not fulfill all the requirements of the AOAC INTERNATIONAL method validation guidelines because of limited material and laboratories avail-

able, it is recommended that AOAC INTERNATIONAL consider approval of this method.

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