Detection of Ricin Contamination in Liquid Egg by Electrochemiluminescence Immunosorbent Assay

David L. Brandon, Anna M. Korn, and Lily L. Yang

Abstract: A monoclonal antibody-based electrochemical luminescence method was developed for detecting and quantifying ricin in liquid egg, with a limit of detection of 0.2 ng/mL. Because this highly toxic protein, present in the seeds of *Ricinus communis* (castor), has been used for intentional poisoning in the past, it is important to have sensitive and reliable analytical methodology to detect ricin in food matrices such as liquid egg. The detection of this quantity of pure or crude ricin spiked into commercial samples of liquid egg provides approximately 50000-fold greater sensitivity than required to detect a toxic dose of ricin (>1 mg) in a 100 g sample.

Keywords: biothreat, castor, electrochemiluminescence, monoclonal antibody, ricin

Practical Application: Because ricin has been used for intentional poisoning, there is a need for analytical methodology to detect ricin in food matrices to assure a safe food supply. Using monoclonal antibodies to ricin developed in our laboratory, we explored an assay readout system known as electrochemiluminescence. This technique afforded sensitive and specific analysis of ricin intentionally added to liquid egg and could potentially be used to monitor egg-based vaccine production.

Introduction

Ricin and the related *Ricinus communis* agglutinin-1 (RCA-1) are toxic storage proteins of the seeds of the castor plant (reviewed by Audi and others 2005; Lord and Spooner 2011). These proteins are also found in the byproducts of the industrial production of castor oil and must be removed or inactivated before the extracted castor mash can be utilized for products such as animal feed (Chen and others 2006; Madeira and others 2011). Because ricin has been used maliciously in the past and has been found at a number of locations as a result of apparent criminal activity (CDC 2003; Schieltz and others 2011), it is important to have sensitive methods for detecting ricin and marker compounds associated with crude ricin preparations. As an alternative to animal models for quantifying toxins, *in vitro* tests, including immunochemical methods, can be used to quantify relevant structural determinants or enzymatic activities and provide essential analytical data to assure food safety (He and others 2008, 2010; Lumor and others 2011). The reported methodologies include various immunoassay formats and devices (for example, Poli and others 1994; Shyu and others 2002; Brandon and Hernlem 2009), activity assays (Hale 2001; He and others 2008), and array and sensor technologies (Feltis and others 2008; Garber and others 2010) for ricin. In addition, assays that measure compounds found in crude preparations of the toxin, such as castor DNA (He and others 2007a, 2007b) and ricinine (Darby and others 2001), offer additional means of detection and forensic attribution. Although immunoassay is most commonly encountered as enzyme-linked immunosorbent assay (ELISA), electrochemical luminescence (ECL) detection is a promising detection technology that offers amplification and, at an emission wavelength of 620 nm, is less subject to interference from matrix effects. Several ECL methods were reported for ricin, especially in liquid matrices (Garber and O'Brien 2008; Cho and others 2009), and its applicability to ground beef, a complex, solid, fatty matrix, was reported by our laboratory (Brandon 2011).

In this study, electrochemiluminescence was evaluated as a detection method for ricin in liquid egg samples. Multiwell ECL plates were used, coated by adsorption with a single ligand (a mouse monoclonal antibody [mAb]), analogous to standard 96-well ELISA plates, and used to detect pure and crude ricin spiked into egg samples.

Materials and Methods

Buffers

Phosphate-buffered saline (PBS, 5 mM Na phosphate, 0.15 M NaCl, pH 7.0) and PBS with 0.05% Tween®-20 (Sigma-Aldrich, St. Louis, Mo., U.S.A.) were used as diluents and wash solutions, as described below. Dilutions of antibody reagents were prepared in PBS-Tween containing 10 mg/L bovine serum albumin (BSA) and 0.01% thimerosal as preservative (BSA-PBS-Tween). Galactose (100 mM) was included in solutions used for ricin analysis to minimize binding of ricin to antibodies or matrix via its lectin sites.

Toxins

Ricin and RCA-1 were obtained from Vector Laboratories (Burlingame, Calif., U.S.A.) and crude ricin was prepared from castor seeds as described previously (Brandon 2011), and had 2.4 mg/mL ricin.

MS 20111185 Submitted 10/1/2011, Accepted 12/22/2011. *Authors are with Foodborne Contaminants Research Unit, Western Regional Research Center, USDA Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710, U.S.A. Direct inquiries to author David L. Brandon (E-mail: david.brandon@ars.usda.gov).*

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Egg analysis

Sample preparation

Shell eggs. Egg shells were cracked and, when fractions were desired, separated into yolk and white fractions. Liquids were collected into tared beakers and were homogenized using Omni homogenizer Model GLH-01 (Omni International, Kennesaw, Ga., U.S.A.) with disposable generator probes, at speed setting 8, for 3 periods of 15 s. Aliquots (2.5 mL) of liquid fractions were then dispensed into conical centrifuge tubes.

Liquid egg samples. Three commercial products were used in this study. Each was pasteurized and homogenized. (1) Citrated liquid egg contained 0.1% citric acid (Challenge Dairy, San Leandro,

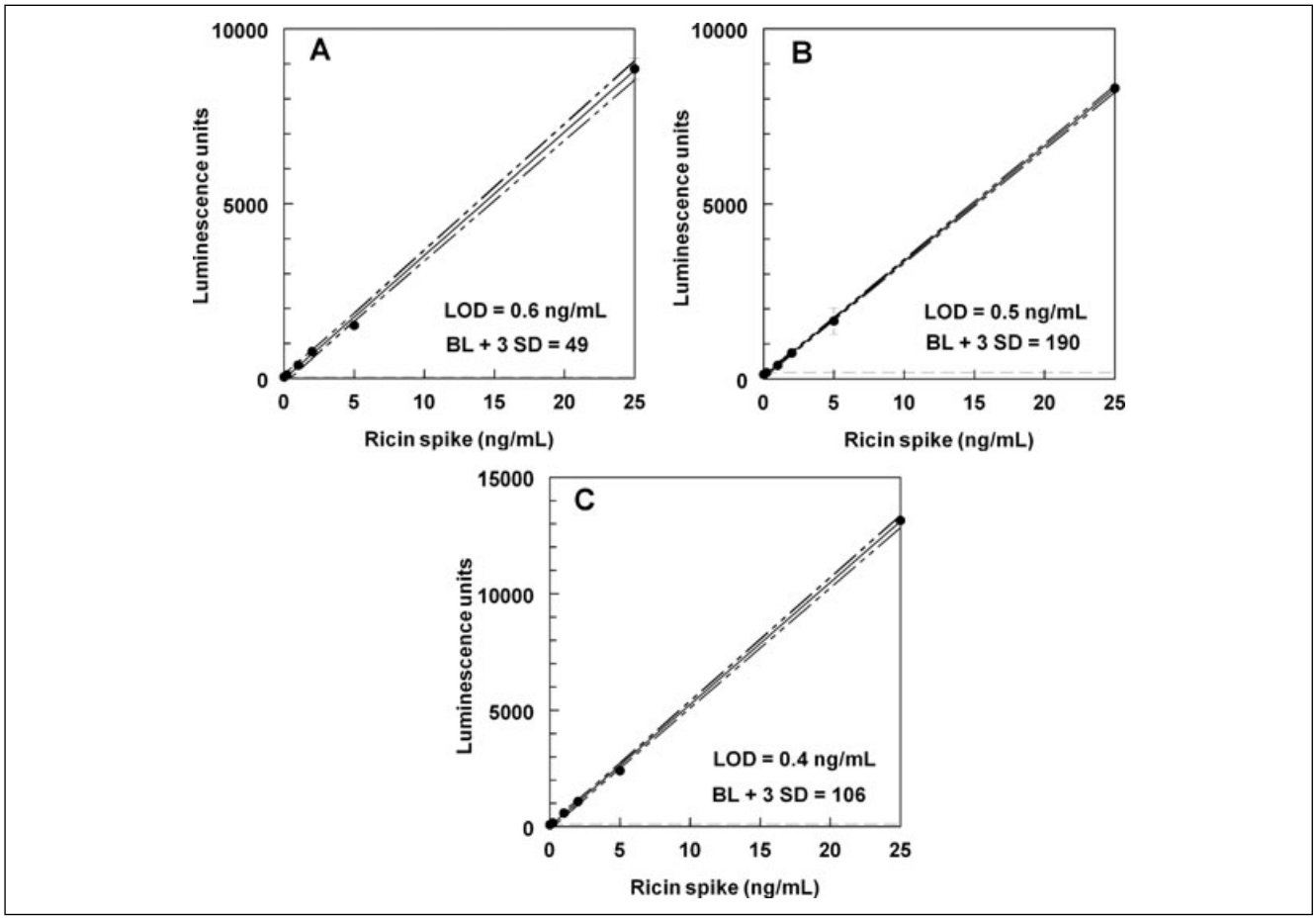

Figure 1–Determination of the lower LOD in 3 egg matrices by ECL assay. Standard ECL plates were coated with mAb 1443 and the sandwich was completed with $Ru(bpy)_3$-mAb 1795. In this example, whole liquid egg (A) and manually separated fractions—yolk (B) and egg white (C) from conventional shell eggs were spiked with various levels of ricin and diluted to a final factor of 1:6.

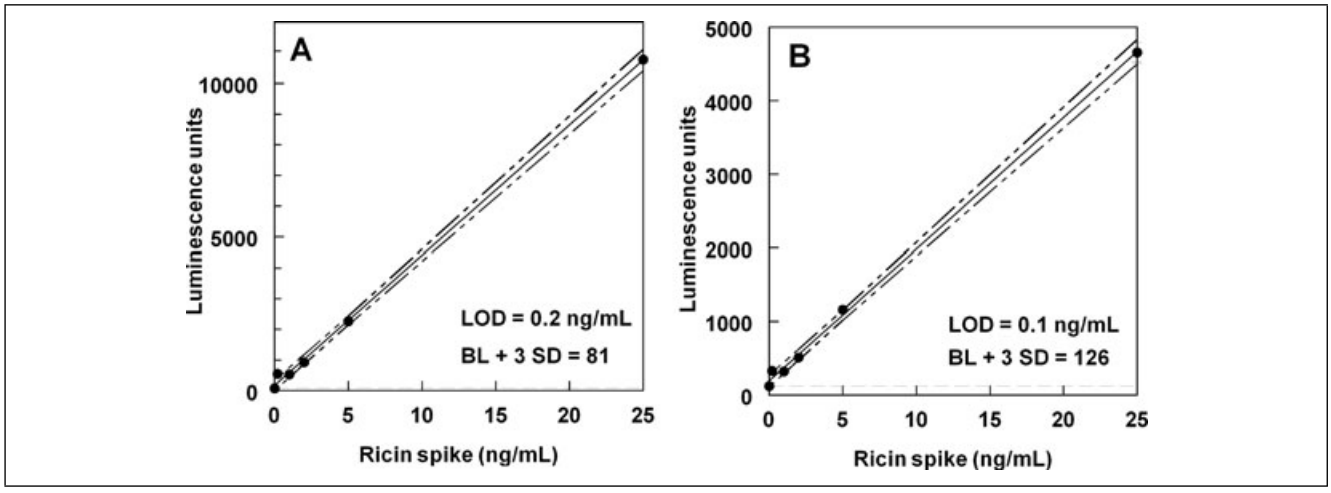

Figure 2–Determination of the lower LOD in whole liquid egg prepared from conventional shell eggs in different ECL sandwich immunosorbent systems. (A) Standard ECL plate coated with mAb 1506. (B) Streptavidin ECL plate, with biotinylated mAb1443 for capture. Assays used $Ru(bpy)_3$-mAb 1795 for detection.

Calif.). (b) Phosphated liquid egg contained monosodium phosphate (Liquid Reddi-Egg, Nulaid Foods, Inc., Ripon, Calif.). (c) Aseptically packaged liquid did not have additives (No MSP Liquid Reddi-Egg, Nulaid) and was dispensed aseptically into sterile 50 mL conical centrifuge tubes. Each of these products had a 2 mo shelf life.

Spiking and analysis. Samples were spiked with ricin preparations using working dilutions from stocks (0.1 to 1 μg/mL). For analysis, 0.5 mL of 0.5 M galactose was added to each aliquot of liquid egg (2.5 mL), with further dilution in BSA-PBS-Tween solution, containing 100 mM galactose (BSA-PBS-Tween-gal). In some analyses, ionic strength was increased by using NaCl at 2- or 3-fold higher concentration (0.3 or 0.45 M). Samples were analyzed at 1:6 final dilution.

Table 1–LODs for ricin in liquid egg samples[a].

	LOD[b] (ng/ mL)	Average cov (%)	Luminescence of positive and negative controls (units $\times 10^{-3}$)	
			+	−
	0.1	7.7	24.5	0.11
	0.5	3.8	20.6	0.13
	0.4	1.9	26.4	0.071
	0.1	2.4	21.0	0.073
	0.1	1.4	20.9	0.065
	0.1	1.9	20.4	0.12
Mean ± SD	0.22	3.2 ± 2.4	22.3 ± 2.5	0.095 ± 0.028

[a]Obtained with $Ru(bpy)_3$-mAb 1443 and capture mAbs used in assays described in text.
[b]Because the lowest spike was 0.1 ng/mL, detection limits < 0.1 and the SD cannot be stated.

Table 2–Blank values for egg samples in ECL immunoassay[a].

Sample[b]	Mean	SD	*n*	Range	
				Minimum	Maximum
1a	80000	20000	2	60000	100000
2a	69	30	5	45	130
2a (yolk)	96	28	4	64	130
2b	88000	85000	4	13000	180000
3a	2110	4490	9	130	14000
1b	214	138	9	67	450
3b	1870	1610	8	220	5040
2c	108	53	8	57	190
1c	78200	10600	8	50	302000

[a]Values in luminescence units.
[b]Samples are whole liquid egg, diluted 1:6, except as noted. Type 1 samples were marketed as conventional eggs; type 2, cage-free; type 3, organic.

ELISA plates

Colorimetric ELISAs were performed on Immulon® 4HBX plates (Dynex, Chantilly, Va., U.S.A.). Plates were coated with proteins at 5 μg/mL, then blocked with BSA-PBS-Tween, treated with 2% sucrose, dried, and stored desiccated as described previously (Brandon and Hernlem 2009).

ECL plates

For ECL immunosorbent assays, 96-well standard bare plates (Cat. Nr L15XA-3) and streptavidin (SA)-coated plates (Cat. Nr L15SA-2) were obtained from Meso Scale Discovery ([MSD], Gaithersburg, Md., U.S.A.). Coating conditions were in accordance with manufacturer's recommendations, with detailed protocols for coating, washing, stabilization, and storage established in preliminary studies (Brandon 2011). Care was taken to avoid or remove air bubbles in all steps in order to achieve reproducible coating and assay development, with 2% to 3% coefficients

Table 3–Recovery of ricin spike from commercial liquid egg samples.

Commercial process/packing	Recovery[a] (%)	*n*
Citrated	79 ± 6.5	6
Phosphated	74 ± 5.9	6
Aseptic	70 ± 6.8	6
All samples	74 ± 6.8	18

[a]Mean ± SD for samples with 25 ng/mL spike.

Table 4–Recovery of ricin from liquid egg fractions[a].

Liquid egg fraction	Recovery[b] (%)		
	mAb 1443	mAb 1506	b-mAb 1443
Whole	63 ± 6.5	75 ± 2.2	73 ± 3.8
Yolk	56 ± 7.6	65 ± 7.5	69 ± 2.4
White	94 ± 7.5	115 ± 6.0	74 ± 4.2

[a]ECL assays used $Ru(bpy)_3$–1795 for detection. Capture mAbs 1443 and 1506 were directly coated by adsorption and biotinylated mAb 1443 was immobilized via streptavidin. Samples spiked at 2, 5, 10, and 25 ng/mL.
[b]Data are mean ± SD, $n = 4$.

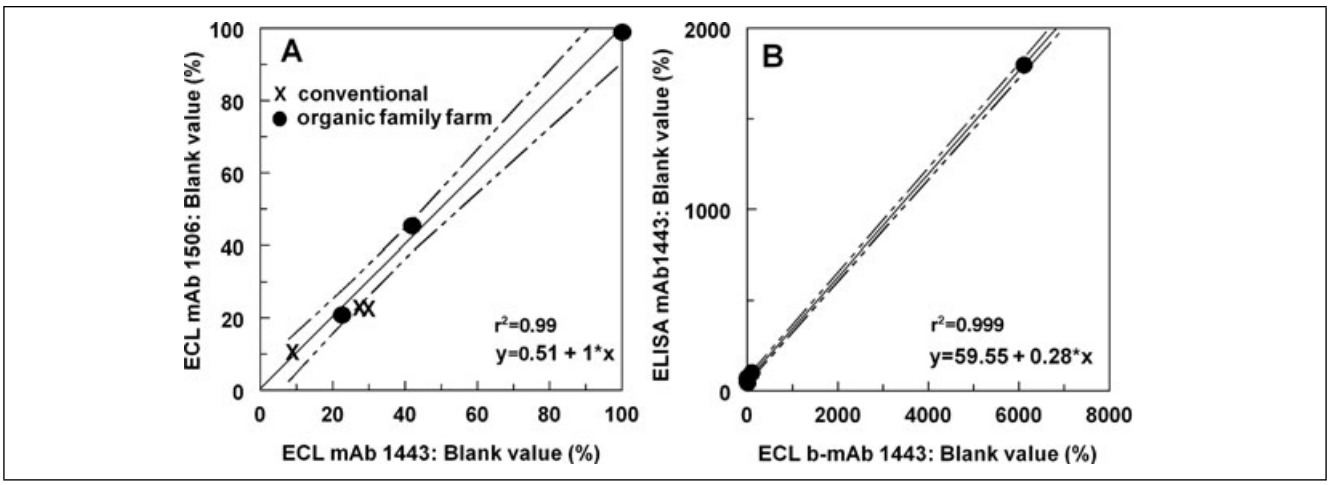

Figure 3–Comparison of blank values (0 spike level) in different assays. (A) Correlation of ECL assays conducted on standard plates, coated with 2 different mAbs. Two different sources of eggs produced similar blanks in the 2 assay systems (normalized as percentage of the highest blank). (B) The correlation of ELISA and ECL assay on streptavidin plates. The point in the upper right-hand corner of the plot represents a sample with unusually high blank values in both assays.

of variation (cov) among well replicates for standards. Standard plates were coated as ELISA plates, but with 50 μL of mAb at 2 μg/mL in PBS, 4 to 16 h. Following blocking, they were used immediately or stabilized and stored. Streptavidin plates were coated with biotinylated mAb at 1 μg/mL, then blocked with 150 μL/well of 30 mg/mL BSA in PBS for 1 h on the day of assay.

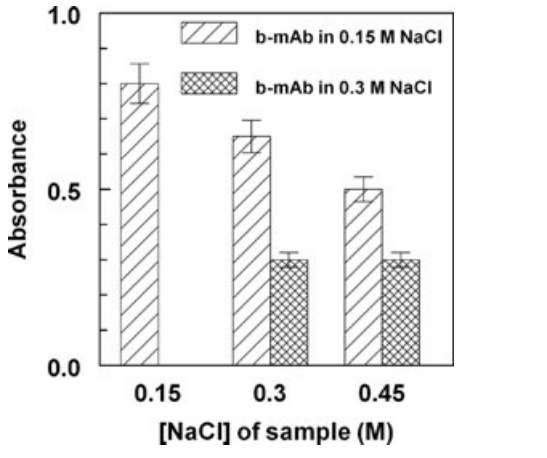

Figure 4–Effect of ionic strength on egg blank values. Sandwich ELISA using mAb 1797 and biotinylated mAb 1655 was conducted under varying conditions of ionic strength. The assay blanks with a typical high-background whole liquid egg sample are shown. The control was conducted under standard conditions using buffers containing 0.15 M NaCl. The effect of having sample or sample and biotinylated mAb in higher ionic strength buffers ([NaCl] = 0.3 or 0.45 M) is shown.

Antibodies and conjugated antibodies

mAbs. Antibodies were prepared, purified, characterized, and biotinylated, as described previously (Brandon and Hernlem 2009). MAbs are designated by the corresponding hybridoma clone numbers. This study utilized mAbs 1506 and 1443 for capture on standard ECL plates and biotinylated mAb (b-mAb) 1443 for capture on SA plates. ELISA utilized the following mAb pairs for capture and detection: mAb 1443, b-mAb 1795 (100 ng/mL); and mAb 1797, b-mAb 1655 (400 ng/mL).

Conjugates for ECL assays. Tris(2,2′-bipyridyl)ruthenium (II) (Ru[bpy]$_3$)-conjugates of antibodies were prepared as described previously (Brandon 2011), with input ratios for conjugation of 12 to 16 mol ester/mol mAb and incorporation of ruthenium label of 5 to 7 mol/mol mAb. Ru[bpy]$_3$-mAb 1795 was utilized at 1 μg/mL.

Data analysis. Plates were imaged using controller software (MSD Data Analysis Toolbox v. 3.0) and data exported to spreadsheets for analysis. This software also enabled images to be spot-checked visually to verify evenness of well coating in preliminary experiments. Standard curves were plotted using a 4-parameter logistic model and the lower limit of detection (LOD) was determined graphically, as described previously (Brandon 2011).

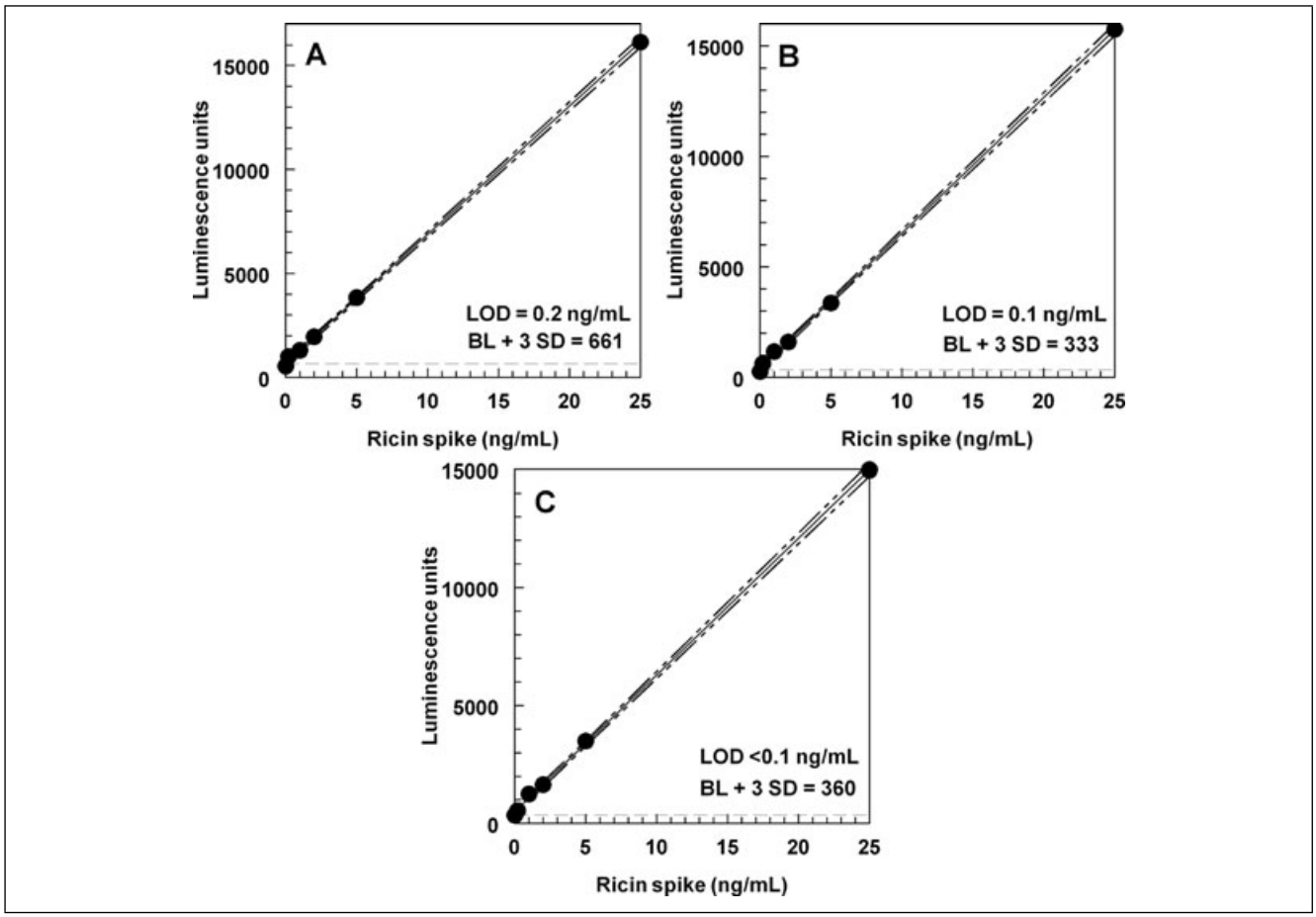

Figure 5–Dose-response and LOD determination in 3 commercial liquid egg samples. Analyses of (A) citrated, (B) phosphated, and (C) aseptically packaged liquid egg samples in ECL immunosorbent analysis using mAb 1506 and Ru(bpy)$_3$-mAb 1795.

Results and Discussion

A typical analysis of shell egg fractions is shown in Figure 1. The results of both ELISA and ECL indicated that analysis of ricin was practical using these techniques. Figure 2 shows the results of analyzing samples with different combinations of mAbs, or with streptavidin-coated plates, using biotinylated mAbs. Blank values were typically in the range of 100 to 200 luminescence units, compared to buffer blanks which averaged 50 to 100 units. The LODs for samples averaged 0.22 ng/mL (Table 1). Because 0.1 ng/mL was the lowest spike level used, we could not determine LODs lower than that level.

In repeating the analyses in over 50 samples, we encountered some samples that produced high blanks (500 to 100000 luminescence units). This result led to a more thorough analysis of the high blanks sometimes encountered, as shown in Table 2. Three sources of grade A eggs were used, based on the package labeling used by local supermarkets in Albany and Berkeley, Calif.: conventional eggs, cage-free, and organic (USDA Agricultural Marketing Service 2010). A previous report of application of a commercial ELISA to staphylococcal enterotoxins (SEs) in eggs and egg products noted false-positive readings found in fertilized eggs (Bennett 2008). We analyzed a subset of the organic eggs that were marketed as "fertilized," but the results were indistinguishable from other samples (data not shown). Blanks were compared in several assay formats, to ascertain whether the high blanks were found in each of them. Figure 3 shows that the high blanks were consistent for 2 mAbs tested and for both ELISA and streptavidin-coated ECL plates.

Bennett (2008) also provided evidence that suggested the Fc regions of the capture antibody was responsible for the false-positive results in commercial ELISA test kits for SEs in egg samples. We analyzed blank values for egg samples in ELISA using Fab and $F(ab')_2$ fragments of 2 ricin-specific mAbs (1443 and 1795), but found that the blanks remained high for these samples. We also explored the possibility that presumptive matrix/antibody interactions leading to high blanks could be altered by raising the ionic strength. Blank values were lowered by about 25% at 0.3 M NaCl (Figure 4), and subsequent analyses were performed with samples and mAbs in BSA-PBS-Tween-gal at 0.3 M NaCl. It should be noted that other approaches to reducing sample backgrounds have been used in analysis of foodborne contaminants, such as concentration of ricin spiked into milk samples using immunomagnetic beads (He and others 2011) and conducting ELISA under nonequilibrium conditions that mitigate matrix effects (Oplatowska and others 2011).

Figure 5 shows the application of the assay methodology to analysis of ricin spiked into commercial liquid egg samples. The blanks ranged from 270 to 550 units, about 2- to 4-fold higher than for individual low-blank egg samples. Table 3 and 4 summarize LOD and recovery data for commercial liquid egg samples. A typical analysis of crude ricin spiked into liquid egg is illustrated in Figure 6. The LODs obtained for crude ricin were similar to those obtained for purified ricin. This method thus provided sufficient and rapid screening for pure or crude ricin levels in the range of 0.1 to 0.2 ng/mL.

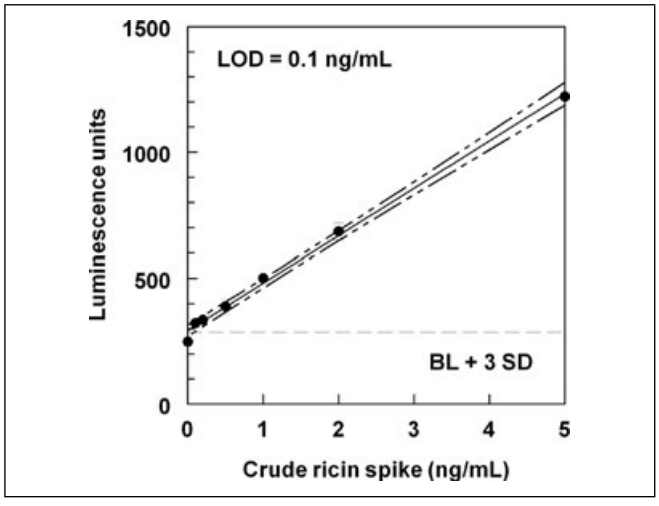

Figure 6–Dose-response and LOD determination for crude ricin in liquid egg. The graph shows the results of analysis of phosphated liquid egg spiked with crude castor at 6 levels from 0.1 to 5 ng/mL ricin and analyzed using the mAb 1443/$Ru(bpy)_3$-mAb 1795 ECL system.

Conclusions

The results indicate that ECL provides a useful analytical technique for ricin in liquid egg samples. Although high blanks were encountered for occasional liquid egg samples prepared in-house from 1 to 3 shell eggs, commercial liquid egg products are prepared from thousands of shell eggs in a single batch. In such samples, the blank values represent the average of many individual eggs. Therefore, it is not surprising that blanks were in the range of 200 to 500 luminescence units, higher than the 100 to 200 units for typical low-blank individual eggs, but much lower than high-background outliers found among shell eggs, which often had blanks of many thousands of units. It seems likely that ELISA and ECL assays in the liquid egg matrix could be further improved with judicious manipulation of conditions that alter protein–protein or protein–lipid interactions. However, ECL provided adequate sensitivity for food defense screening through determination of pure or crude ricin in contaminated liquid egg. The format is robust and multiplexing is possible, enabling multivalent testing for analytes in multiple samples within single-plate assays, in either 96-well or 384-well formats.

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