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Development of a Fluorescent Latex Microparticle Immunoassay for the Detection of Staphylococcal Enterotoxin B (SEB)

MARJORIE B. MEDINA†

Microbial Biophysics and Residue Chemistry Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038

Staphylococcus aureus enterotoxin B (SEB) is a highly heat resistant enteric toxin with a potential as a biothreat agent. A sensitive method for the detection of staphylococcal enterotoxins is needed for food safety and food defense monitoring. The objectives of this research were to develop a competitive fluorescent immunoassay with detection of SEB below toxic levels of 1 ng/mL and to minimize sample preparation. Anti-SEB was immobilized onto carboxylated polystyrene microparticles, and SEB was labeled with fluorescein isothiocyanate (FITC). The concentrations of these reagents were optimized for the detection of SEB below 1 part per billion (1 ng/mL), and other assay conditions (sample volumes and incubation periods) were optimized. Drinking water and milk samples were spiked with 0.125−10 ng/mL SEB and were equilibrated overnight prior to analysis. The water and milk samples were directly analyzed, but heating the milk samples for 10 min at 90 °C improved the assay performance. SEB in samples bound with the anti-SEB linked to the latex followed by the competitive binding of SEB−FITC tracer. The excess, unbound tracer was separated by centrifugation, and the fluorescence density of the supernatant was measured. SEB was detected at levels as low as 0.125 ng/mL in drinking water and 0.5 ng/mL in whole milk. This fluorescent latex particle immunoassay will be utilized for the detection of SEB in various foods matrices.

KEYWORDS: Staphylococcal enterotoxins; fluorescent; immunoassay; SEB; drinking water; milk; latex particle

INTRODUCTION

Rapid and inexpensive methods are needed to detect trace levels of enterotoxins produced by *Staphylococcus aureus* organisms. *S. aureus* produces enteric toxins that cause a major foodborne gastroenteritis. Under temperature-abused conditions, the organism can grow in foods of animal origin. Heat processing and normal cooking temperatures can inactivate or kill the bacterial cells, but the enterotoxins (SE) are heat stable and are resistant to these cooking and heating temperatures (*1*, *2*). Staphylococcal enterotoxins, A, B, C1, C2, C3, D, and E (*1*), H (*3*), I and G (*4*), and J (*5*), have been identified. However, *S. aureus* toxins A, B, C, and D are the most common in foods, and SEA is the most prevalent in food-poisoning outbreaks.

An estimated 185000 cases of staphylococcal food poisoning were reported by Mead et al. (*6*). The Centers for Disease Control and Prevention (CDC) reported 4870 cases in a passive surveillance from 1992 to 1997, and 487 cases were reported from outbreaks. In all cases, the toxins were transmitted through food. SEB is a potent gastrointestinal toxin and heat resistant, and this is also a potential biothreat agent (*7*). The toxins have molecular weights ranging from 27000 to 34000, and SEB has a molecular weight of 28336 with an isoelectric point (p*I*) of 8.6 (*8*). The relative thermostabilities of these toxins are SEC > SEB > SEA (*9*). The minimum level of enterotoxin to cause gastroenteritis syndrome in humans was ≈1 ng/g or ng/mL of food (*10*, *11*), whereas Newsome (*2*) and Tatini et al. (*12*) reported <1 μg of toxin ingestion. Methods with detection at or below 1 ng/g (1 part per billion) are desired, and testing for both organism and toxin can ensure the safety of processed foods.

Immunochemical assays were developed and utilized in the past 20 years for the detection of staphylococcal enterotoxins. The principles and performance of these immunological methods were described in refs *13*−*15*, and the biological and PCR-based methods were further reviewed (*15*). ELISA-based commercial methods were recently reviewed (*16*). In the review, the specificities and relative sensitivities of four immunoassay kits, TECRA-SET VIA (Tecra International, New South Wales, Australia), VIDAS SET and VIDAS ELFA systems (BioMerieux, Marcy l'Etoile, France), and Transia Tube SET (Diffcamb AB, Vastra Frolunda, Sweden), were evaluated. The TECRA,VIDAS SET, and Transia detected SE in foods with natural contaminants from food-poisoning outbreaks. However, TECRA and TRAN-

† Telephone (215) 233-6436; fax (215) 233-6559; e-mail mmedina@errc.ars.usda.gov.

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SIA had false-negative results in cheese samples spiked with ≤1 ng/g SE, whereas TRANSIA showed false-negative results in chicken and false-positive results in water chestnuts. The VIDAS ELFA (enzyme-linked fluorescent assay) system detected effectively the renatured toxins from contaminated canned mushrooms. The enzyme immunoassay (Transia-Diffchamb) detected 1 ng/mL enterotoxin in milk and 0.5−0.8 ng/g in mushrooms, ravioli, and meat (*17*). A lateral flow device, a "hand-held assay", with detection of SEB at <1 ng/g in custard was reported (*18*). Two commercial immunoassays (RIDASCREEN and TRANSIA TUBE ELISA assays) were also tested, resulting in detection in the 0.5−2.5 ng/g range. The development of a fluorescent immunomagnetic flow cytometry for the detection of SEB detection in milk had a sensitivity of 0.25 ng/mL (*19*). SEB was detected at 0.1−100 ng/mL in skim milk samples at 1:20 dilution (5%) using an immunomagnetic−elctrochemiluminescent system by Origen (*20*). This is equivalent to detection in undiluted samples at 2−2000 ng/mL. Medina (*21*, *22*) reported a method using a surface plasmon resonance biosensor (Biacore) detecting SEB at 2.5 ng/g in ham using a sandwich assay format and at 0.78−50 ng/mL in milk using a competitive assay format. These reports (*21*, *22*) also summarized the utilization of other biosensor techniques for the detection of SE in foods.

The objectives of the current study were to develop methods alternative to the commercial assays and the highly expensive biosensor systems; to optimize the preparation of the tracer and covalently linked antibody; to develop methods that can utilize laboratory-prepared reagents for routine monitoring of SE in foods with sensitivity at the toxic levels (<1 ng/mL or 1 ng/g); and to minimize sample preparation. The fluorescent latex microparticle immunoassay (FLMIA) conditions developed will be utilized for the development of multitoxin detection in the food systems.

MATERIALS AND METHODS

Equipment and Reagents. Staphyloccoccal enterotoxin B (SEB) and affinity-purified polyclonal sheep anti-SEB were obtained from Toxin Technology (Sarasota, FL). Polystyrene carboxylated latex microparticles (0.78-Mm, COOH/2, 10% w/v) were from Bangs Laboratories (Fisher, IN). Fluorescein isothiocyanate (FITC), Hepes (free acid), ethylenediaminetetraacetic acid (EDTA), sodium phosphate, sodium azide, Tween 20 and Brij surfactants, and Sephadex G-10 were obtained from Sigma Chemicals (St. Louis, MO). Ethanolamine was from Biacore Inc. (Piscataway, NJ); bovine γ-globulin standard and Slide-a-Lyzer with 10000 MW cutoff and 1−3 mL capacity were from Pierce (Rockford, IL); and silica gel G (scored 10 × 10 cm, 250 μL thickness) Uniplate TLC plates were from Analtech (Newark, DE). A Tecan Safire2 multimode microplate reader (Grodig, Austria), Biotek microplate reader (EL 312; Winooski, VT), Tomy MTX50 centrifuge (Peninsula, Laboratories, Inc., Belmont, CA), Cary Eclipse fluorescence spectrophotometer (Varian, Walnut Creek, CA), Microfluor microtiter wells (Dynatech Laboratories, Chantilly, VA), and IKA Vibrax VXR shaker (Fisher Scientific, Pittsburgh, PA) were used. Silica gel G (2.5 × 10 cm) TLC plates were prewashed in methanol and dried at 85 °C in an oven for 15−20 min.

Buffer Reagents. The latex storage buffer consisted of 10 mM HBS (pH 7) storage buffer containing 3.4 mM EDTA, 150 mM NaCl, 0.1% w/v sodium azide, 0.015% Brij surfactant, and 0.1% BSA (protease free). Latex assay buffer 1 was 10 mM HBS buffer (pH 7) [Hepes-free acid 1.19 g; 5 mM EDTA (0.182 g; 0.9% NaCl (0.5 g); 0.1% BSA (0.1 g); 0.05% Tween 20 (0.05 g); 0.1% sodium azide (0.1 g) in 100 mL], and latex assay buffer 2 was the same as latex assay buffer 1 without BSA. FITC labeling buffer consisted of 10 mM phosphate buffer (pH 8). Dialysis and column purification buffer was 10 mM phosphate buffer (pH 7).

FITC Labeling of SEB. SEB (1 mg) was dissolved in 1 mL of 10 mM phosphate buffer (pH 8) in a polypropylene vial and mixed with a magnetic stirring bar. FITC (1.55 mg) was dissolved in 1.14 mL of 10 mM phosphate buffer (pH 8) (1.36 mg/mL concentration). To ease dissolution, the FITC solution was sonicated until the powder was dispersed. The FITC solution (1 mL) was added dropwise to the SEB solution and mixed gently for 4 h at room temperature. The final concentrations were 0.5 mg/mL SEB (0.0177 mM) and 0.68 mg/mL (1.74 mM) for FITC. The reaction was allowed to continue overnight at 4 °C. The progress of derivatization was screened with silica gel G TLC. Using a microcapillary pipet, a 1 μL aliquot of the reaction mixture was drawn and applied on the silica gel G TLC plate. The spot was dried with a stream of nitrogen and developed in a pre-equilibrated 10 mL developing solvent consisting of chloroform/methanol/water (v/v/v) for 10 min in a 10.5 × 5 cm or 13 × 5 cm jar.

Purification of the Derivatized SEB. The labeled SEB was purified in two steps by dialysis followed by column chromatography. The reaction mixture was transferred to a Pierce Slide-a-Lyzer, and the tube was rinsed with 0.5 mL of 10 mM phosphate (pH 7) buffer and injected into the dialyzer. The mixture was dialyzed in 1 L of 10 mM PBS (pH 7.0) for 3 days (72 h) at 4 °C, changing buffers in the morning and afternoon. Aliquots of the dialysate were checked with TLC plates (2.5 × 10 cm) prewashed in methanol and activated in an 85 °C oven for 20 min. The plates were developed in chloroform/methanol/water (4:4:1 v/v/v) for 10 min (*23*). The dialyzed sample was removed with the 3 mL syringe and transferred to calibrated tubes. The Slide-a-Lyzer was rinsed with 0.5 mL of phosphate buffer and added to the rinse buffer of the dialyzed sample. The total volume was measured and adjusted to 5 mL with the phosphate buffer, and 2.5 mL was transferred to the Sephadex G-10 column. A 4 g Sephadex G-10 gel was swollen in phosphate buffer (pH 7) overnight or longer. The gel was transferred to an Econo-Pac column (Bio-Rad, Richmond, CA) fitted with a two-way luer lock stopcock (Bio-Rad) at the bottom. The column was filled with the gel to ≈1.5 cm diameter and 5 cm height. The gel was equilibrated with phosphate buffer, and a frit was placed over the top of the gel, and the column was further equilibrated with buffer. After the buffer had been allowed to percolate, the column was locked. A 2.5 mL aliquot of SEB−FITC derivative was applied. The first 2.5 mL of eluent (void volume) was collected and discarded. The derivative was eluted with 5 × 1 mL and 5 × 0.5 mL of 10 mM PBS buffer. Each fraction was diluted in PBS buffer (1:10), and the fluorescence was measured at 485 nm excitation and 520 nm emission (gain = 100) using a Cary Eclipse spectrofluorometer. The relative fluorescence units (RFU) were plotted against the fraction number. Each fraction was also screened with TLC by applying 1 μL of the undiluted fractions with high fluorescence and 2 μL for fractions with lower fluorescence density. The fractions in the first peak were pooled for use as the assay reagent. If there was still a high ratio of free FITC, a 2.5 mL mixture was further purified through a Sephadex G-10.

Immobilization of Anti-SEB−IgG to Latex Particles. Anti-SEB (2 mg) was diluted in 6.6 mL of 10 mM sodium acetate buffer (pH 4.5). Aliquots of 2 mL of anti-SEB were transferred to 3 × 4 mL polypropylene tubes. The carboxy latex particles [polystyrene carboxylated latex particles (0.78 mm, COOH/2, 10% w/v)] were sonicated with 10 pulses at 75% duty cycle (power setting 3), and 200 μL aliquots of latex particles were transferred into 3 × 10 mL conical polypropylene centrifuge tubes. Equal volumes of EDC (75 mg/mL H_2O) and NHS (11.5 mg/mL H_2O) were mixed, and 50 μL aliquots of EDC/NHS were transferred to each of the three tubes containing the latex particles. The latex and EDC/NHS were gently mixed for 20 min at room temperature to activate the carboxyl groups. Anti-SEB (2 mL of 0.3 mg/mL) was added dropwise with continuous vortex mixing for 60 min at room temperature. The derivatized latex (latex−anti-SEB−IgG) was centrifuged at 8225*g* (10000 rpm) for 5 min at 4 °C. The supernatant was assayed for protein content using a Bio-Rad protein assay reagent and Pierce protein globulin standards and compared with the initial protein concentration. The derivatized latex was resuspended in 2 mL of HBS (pH 7) buffer without BSA or surfactants (Tween or Brij). Ethanolamine (1 M, 50 μL) was added to block the unreacted active carboxyl esters and further mixed for 30 min at room temperature. The latex−anti-SEB was separated by centrifugation at 10000 rpm (8225*g*) for 10 min, and the supernatant was discarded. The latex−anti-SEB was stored in 2 mL of HBS (pH 7) storage buffer containing

Table 1. Protocol for the Detection of SEB[a]

			latex–SEB IgG was added to the 0—10 ppb sample tubes							
Reagents	blank	SEB-F 1:10	0 SEB	0.125 ppb of SEB	0.25 ppb of SEB	0.25 ppb of SEB	0.5 ppb of SEB	1 ppb of SEB	2.5 ppb of SEB	10 ppb of SEB
buffer	150	100								
latex–SEB IgG (μL)			50	50	50	50	50	50	50	50
sample (μL)			50	50	50	50	50	50	50	50
incubate					30 min; mix with a rotary mixer					
SEB–FITC (μL)		50	50	50	50	50	50	50	50	50
incubate					60 min; cover samples with foil; mix					
buffer (μL)	150	150	150	150	150	150	150	150	150	150
total vol (μL)	300	300	300	300	300	300	300	300	300	300

[a] Reagents used: latex immobilized with anti-SEB–IgG (1:5 dilution) and SEB–FITC tracer (1:10 dilution) and spiked SEB in samples from 0.125 to 10 ng/mL (ppb). Microfuge tubes (1.2 mL) were used, and tubes were labeled as shown in the first row.

3.4 mM EDTA, 150 mM NaCl, 0.1% w/v, sodium azide, 0.015% Brij surfactant, and 0.1% BSA (protease free). The latex derivatives were pooled for use in the subsequent assays.

Immunoassay Procedures. Optimization of the SEB−FITC and the latex−anti-SEB was carried out using competitive binding of labeled SEB−FITC and SEB with latex−anti-SEB in buffer. Various dilutions of the reagents, total assay volume, and incubation periods were assessed such as SEB−FITC at 1:5, 1:10, 1:20, and 1:50; latex−SEB anti-SEB (1:5, 1:10, and 1:20); total sample volume of 200, 250, and 300 μL; and incubation periods of 30/30, 30/60, and 45/45 min binding of SEB with latex−IgG and competition binding with SEB−FITC, respectively.

A typical protocol is shown in **Table 1**. After the total volume was adjusted to 250 or 300 μL, the microfuge tops were cut off and the latex−IgG−SEB−SEB−FITC complex was separated from the unbound free SEB−FITC by centrifugation at 13500 rpm (14989*g*) for10 min at 10 °C. Aliquots of the supernatants (2 × 100 μL) were accurately transferred to microtiter wells. Using the Tecan Safire 2, the relative fluorescence was measured at 150, 175, and 300 gain using an excitation wavelength of 230 nm and an emission wavelength of 525 nm. The excitation and emission bandwidths were 2.5 and 5.0, respectively, with a 5400 Z factor.

Sample Preparation. Drinking water (tap and bottled water) was spiked with SEB and analyzed after mixing or after overnight equilibration at 4 °C. Whole milk was obtained from a local market and aliquoted for storage at −80 °C for subsequent analysis. The latex assay buffer and milk were spiked with SEB and analyzed after equilibration overnight. The spiked samples were prepared by serial dilution from 10 to 0.125 ng/mL SEB. Alternatively, samples (0.9 mL) were transferred to 4 mL conical polypropylene tubes and spiked with 0.1 mL of SEB (0, 1.25 2.5, 5, 10, 25, 50, and 100 ng/mL) and equilibrated for 1 or 16−18 h at 4 °C. The final SEB concentrations were 0, 0.125, 0.25, 0. 5, 1.0, 2.5, 5, and 10 ng/mL. The samples were analyzed using the protocol described in **Table 1**. In later assays, the spiked milk samples were heated for 10 min at 90 °C and cooled to ambient temperature prior to FLMIA analysis.

RESULTS AND DISCUSSION

Preparation of SEB−FITC Tracer. The TLC results showed bands at the origin indicating FITC labeling of SEB. Dialysis removed excess FITC, and column chromatography further separated the tracer from FITC. A typical separation of SEB−FITC is shown in **Figure 1**. Fractions containing SEB−FITC in the first peak were pooled. Typically, the SEB−FITC fractions were contained in a total of 5 mL of pooled fractions. A single chromatographic cleanup of 0.5 mg/mL SEB did not show significant binding to the latex−IgG, but in a second chromatographic purification, the SEB−FITC bound to the latex−IgG. An SEB−FITC concentration of 0.25 μg/50 μL was utilized in the assay. The SEB and FITC mixture containing 0.5 mg/mL stored for 4 weeks at 4 °C was chromatographically purified. The TLC analysis also showed a higher density of bands near the origin. The tracer was pooled from fractions 2−7 to a total volume of 5.1 mL. Aliquots of 50 μL of 1:10 dilution containing 0.5 μg/50 μL were used in the assay. This tracer showed higher fluorescence density and higher binding to the IgG latex and greater competition with the sample SEB compared to the tracer purified in previous experiments. These results suggest that tracer preparation needs to be optimized as a higher FITC labeling occurred in 4 weeks of storage and without interference with SEB binding to the latex−IgG.

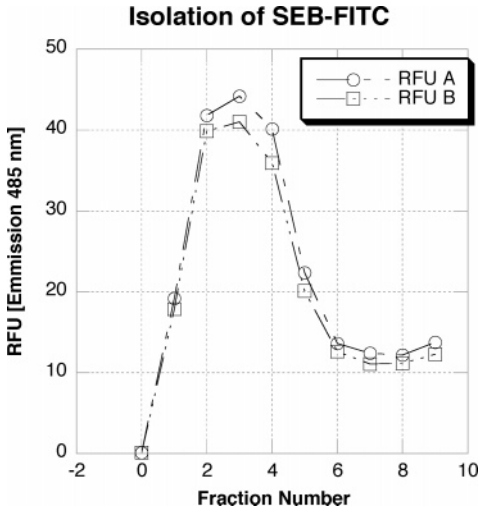

Figure 1. Purification of SEB–FITC through Sephadex G-10. Fractions were diluted 1:10 with phosphate buffer for fluorescence measurement at 485 Em/520 Ex. Fractions 1–8 were pooled, yielding a total volume of 5.1 mL. The SEB–FITC concentration was equivalent to 100 μg/mL. RFU, relative fluorescence unit. RFU A and RFU B are mean fluorescence densities of two readings of duplicate samples.

Table 2. Characteristics of the Latex Microparticles Immobilized with Anti-SEB Immunoglogulin (IgG)

batch	initial IgG concn (mg/mL)	IgG protein load (mg/mL)	% load	IgG concn (μg/50 μL)
1-062305	0.402	0.361	89.8	3.61
2-081205	0.428	0.369	86.1	3.69
3-100305	0.428	0.366	85.4	3.66
4-110205	0.333	0.301	90.4	3.01

Covalent Attachment of IgG to Latex Particles. Table 2 shows a summary of anti-SEB−IgG immobilization. The first three batches of latex particles were immobilized as described in a previous paragraph. This immobilization technique was adopted from our previous study (*23*), but the 30 min immobilization was increased to 60 min with protein uptakes of 71 and 87%, respectively. The latex particles had a mean of

0.365 (± 0.03) mg of IgG protein load when measured against a globulin protein standard. The fourth batch (4-110205) of the latex−IgG had a slightly lower SEB IgG concentration of 0.301 mg of IgG/mL. This batch resulted in a higher immobilization efficiency (90.4%). The higher percent yield may have been due to additional time (30 min) for activation of the latex and additional overnight incubation of the IgG and the activated latex prior to separation of the latex−IgG complex. However, these differences are small; the mean immobilization efficiency was 87.22 (SD 2.2). A 1:5 dilution was utilized in the assays wherein 50 μL contained a mean concentration of 3.65 μg (SD 0.03) of IgG derived from the first three batches. The latex−IgG preparation procedure described had minimal batch-to-batch variability with respect to yield, protein load, antigen−antibody binding, and assay performance.

Fluorescent Latex Microparticle Immunoassay (FLMIA). The assay performance of 1:5, 1:10, 1:20, and 1:50 dilutions of the latex−IgG and 1:5, 1:10, 1:20, and 1:50 SEB−FITC were optimized using SEB-spiked water and buffer samples. The optimum concentrations selected were 1:5 latex−IgG and 1:10 SEB−FITC tracer. A two-step incubation assay format (**Table 1**) was utilized, which allowed trace level detection of analytes. The first step allowed binding of the sample SEB to the latex−IgG, and the second incubation step allowed the tracer to bind to unoccupied binding sites of the IgG. After separation of the anti-SEB−SEB−FITC-latex complex through centrifugation, the fluorescence density of the supernatant, which contained unbound (free) SEB−FITC (tracer), was measured. The fluorescence density of the unbound tracer was directly proportional to the concentration of SEB in the standards or samples. That is, the fluorescence density increased as the SEB concentration increased. Numerous experimental trials were conducted to optimize the assay conditions to detect SEB at the picogram level. The results suggest yields of 50 μL of 3.65 μg of IgG−latex in a 1:5 dilution and 50 μL of 0.5 μg of SEB−FITC (1:10 dilution); 150 μL was the total assay volume; a two-step incubation of 45 min of preincubation followed with 45 min of incubation with SEB−FITC was used. A 150 μL buffer was added to the assay mixture to minimize errors in sampling 2 × 50 μL supernatants containing the free/unbound tracer. Fluorescence density was measured at pH 7; although this pH yielded lower fluorescence density compared to pH 8.5, another step of adjusting the pH is eliminated by using pH 7.

Water Analysis. Tap water and a commercial bottled water were spiked with SEB and equilibrated overnight at 4 °C prior to FLMIA analysis. The SEB−FITC utilized was twice purified through a Sephadex G-10 column, where each 50 μL aliquot contained 0.25 μg of SEB−FITC. **Figure 2A** shows the calibration curves in spiked bottled water. The results show increasing fluorescence density of the free/unbound SEB−FITC in samples containing 0.125−10 ng/mL SEB versus the zero control. With increasing dose of SEB in samples, the tracer (SEB−FITC) was blocked from binding to the latex−anti-SEB binding sites and, therefore, the free SEB−FITC remained in the supernatant. The percent inhibition was determined as follows: (RFU of sample − RFU of 0 control)/RFU 0 control × 100. The results for the analysis of spiked bottled water and tap water are shown in **Figures 2B** and **3**, respectively. In four separate analyses of bottled and tap water, the minimum detection is indicated at 0.125 ng/mL with a mean inhibition of 16.25% (±5.1 SD) and 23.9% (±6.8 SD), respectively. The maximum binding inhibitions to the latex−IgG were 34 and 41% at 5 ng/mL. This assay utilized the HBS storage buffer containing 0.1% BSA and 0.015% Brij surfactant.

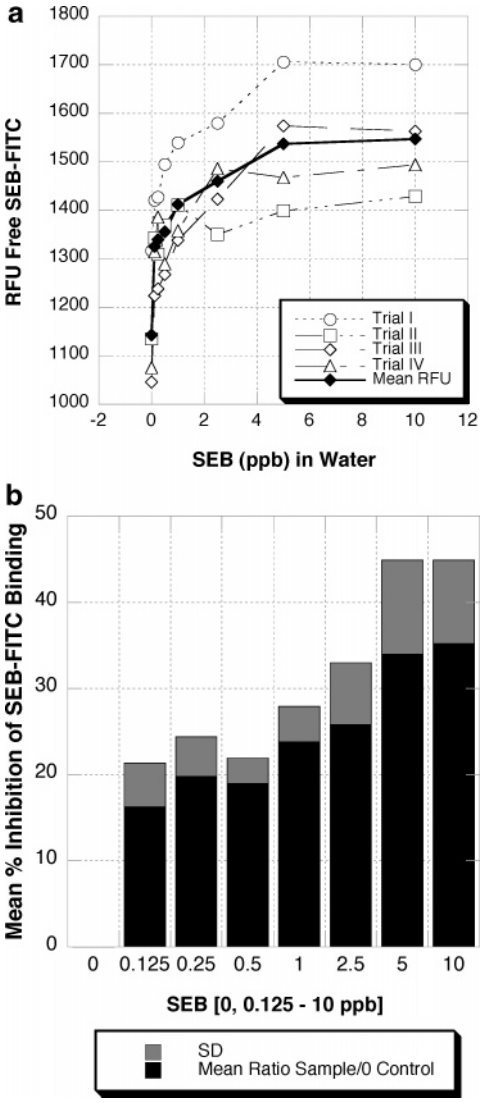

Figure 2. (**A**) Dose–response curves of SEB concentration versus relative fluorescence unit (RFU) in four trials in bottled water spiked with SEB. The fluorescence density of the free/unbound SEB–FITC is directly proportional to the concentration of SEB in samples. (**B**) Bar graphs of percent inhibition of SEB–FITC binding with anti-SEB latex by SEB spiked in the samples. Percent inhibition = [(RFU of samples − RFU of "0" control samples)/RFU of "0" control samples] × 100. These graphs indicate detection of SEB at 0.125 ppb in bottled water.

Milk Analysis. The milk samples were thawed and spiked at 0.125, 0.25, 0.5, 1.0, 2.5, 5, and 10 ng/mL. The spiked samples were equilibrated overnight and directly analyzed following the FLMIA procedure used with water. The direct analysis of the milk samples presented some challenges. Some of the analyses showed that the 0 control samples had higher RFU (of the free/unbound SEB−FITC) than the samples spiked below 1 ng/mL. This was apparently due to the coating of the IgG−latex by the BSA, thus blocking the binding of the tracer (SEB−FITC). In subsequent experiments, BSA was eliminated from the assay by using latex assay buffer 2 (without BSA). Results from this approach using the percent inhibition ratios are shown in **Figures 4** and **5**. Compared to the results from water analysis, the inhibition ratios were lower in the milk samples, and this indicates sample matrix interference due to coating of the antibody binding sites. Proteins in milk (such as casein) have been used to block nonspecific binding sites in some immunochemical assays. To improve performance of the

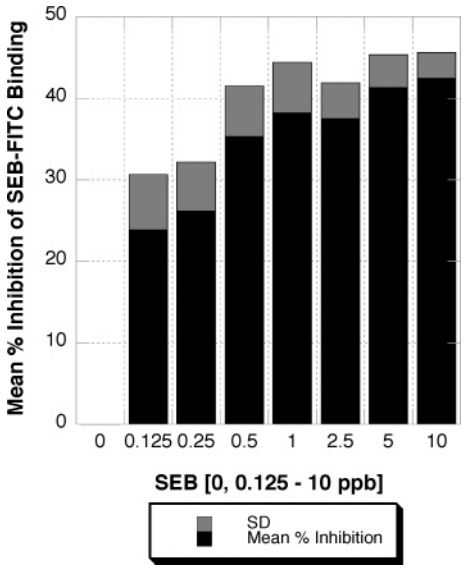

Figure 3. Dose–response graphs of the analysis of SEB spiked in tap water; plot of the mean percent inhibition of SEB–FITC binding with anti-SEB–latex by SEB in the samples. Percent inhibition = [(RFU of samples – RFU of "0" control samples)/RFU "0" control samples] × 100. The percent inhibition is directly proportional to the SEB concentration in tap water samples. These graphs indicate detection of SEB at 0.125 ppb in tap water.

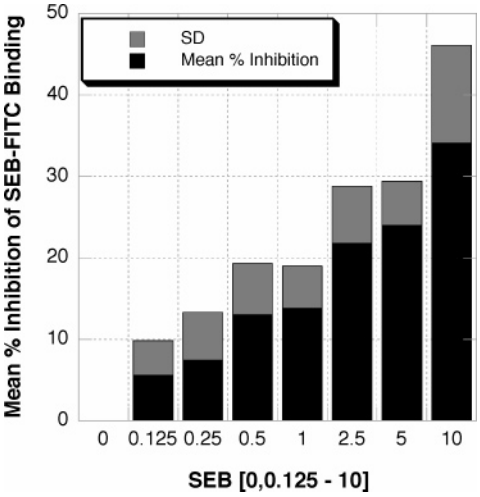

Figure 4. Dose–response graphs of SEB spiked in whole milk; plot of the mean percent inhibition of SEB–FITC binding with anti-SEB–latex by SEB in the milk samples in eight experimental trials. The graph shows a lower inhibition ratio compared to the water samples (**Figures 2** and **3**). In this FLMIA assay, samples containing SEB were preincubated with the latex–anti-SEB prior to addition of the tracer, SEB–FITC.

assay, the format was further modified by preincubating the SEB−FITC with the latex−antibody prior to addition of the milk samples. **Figure 5** shows the relative inhibition of SEB−FITC binding by the SEB in samples. This approach resulted in a more linear response of the assay from 0.125 to 10 ng/mL. The comparison of the mean percent inhibition and the standard deviations of the assays in **Figures 4** and **5** are shown in **Table 3**. The mean standard deviations were 6.28 and 5.67, respectively, whereas the mean relative standard deviations (RSD) were 0.366 and 0.33 for concentrations of 0.25−10 and 0.5−10 ng/mL for assays with sample preincubation. The RSDs were 0.31 and 0.26 for concentrations of 0.25−10 and 0.5−10 ng/mL in assays in which the tracer (SEB−FITC) was allowed to

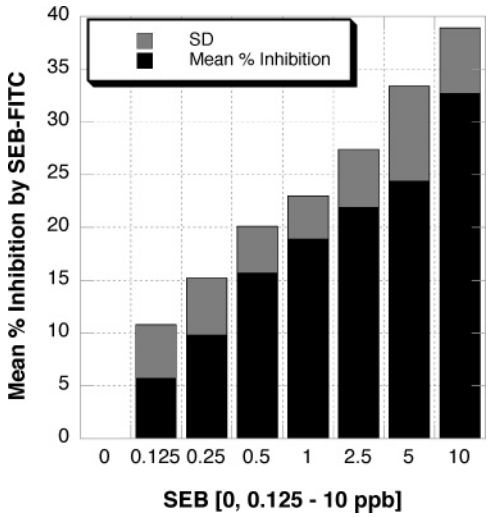

Figure 5. Dose–response of SEB spiked in whole milk versus percent inhibition of SEB–FITC binding to the latex–anti-SEB. In this assay approach, the samples were preincubated with SEB–FITC followed by the addition of milk samples spiked with SEB to minimize the sample matrix interferences. The bar graph shows the mean percent inhibition of SEB–FITC binding to the anti-SEB–latex in five separate trials.

Table 3. Comparison of the Performance of Two Assay Formats in the Inhibition of the SEB–FITC Binding with the Latex–IgG by SEB-Spiked Whole Milk

SEB concn (ppb)	mean % inhibition I[a]	SD[a]	RSD[c]	mean % inhibition II[b]	SD[b]	RSD[c]
0	0.00	0.00	0	0.00	0.00	0
0.125	7.6	3.2	0.42	5.70	5.10	0.89
0.25	**8.2**	**4.5**	0.55	9.80	5.40	0.55
0.5	16.2	6.4	0.39	**15.70**	**4.40**	0.28
1.0	16.9	6.2	0.37	18.90	4.10	0.22
2.5	20.9	7.7	0.37	21.90	5.50	0.25
5.0	25.2	5.2	0.21	24.40	9.00	0.37
10.0	34.7	10.8	0.31	32.70	6.20	0.19

[a] Mean of percent inhibition and standard deviation (SD) from eight assay trials in **Figure 4**. In these assays, the samples containing SEB were preincubated with the latex–anti-SEB prior to the addition of the tracer (SEB–FITC). [b] Mean percent inhibition and standard deviation (SD) in five trials in **Figure 5**, where SEB–FITC was preincubated with the latex–anti-SEB prior to incubation with the milk samples spiked with SEB. The bold figures indicate the minimum concentration higher than 2 × SD of the 0.125 ppb samples. [c] Relative standard deviation = standard deviation/mean.

bind with the anti-SEB before binding of SEB in milk samples, and this format shows an improvement in the assay performance. These results suggest that the assay precision needs to be improved. Measurement of the fluorescence density resulted at times in high variability between readings of duplicate aliquots from the same samples for fluorescence reading only, and this problem may be attributed to the instrument. The latex dispersion may also influence the binding efficiency of SEB and the SEB−FITC. The effects of proteins in the samples may be minimized by heating or precipitation.

These studies demonstrate that the fluorescent microparticle immunoassay (FLMIA) can be used for trace analysis (below 1 ng/mL) of SEB in drinking water (0.125 ng/mL) and whole milk (0.25−0.5 ng/mL). The milk samples spiked with SEB were analyzed directly without sample preparation. The analysis can be completed in <2.5 h for 20 samples. This research also shows the preparation of the tracer and the covalent linking of the antibody to the latex with the cost of reagents (tracer and

anti-SEB−latex) of <$1 per test. This assay can be used for high-throughput analysis. Our future studies will include the analysis of spiked meat and poultry tissues, expand our assays for SEA detection, and streamline sample preparation of food products.

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