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ARTICLE

Simultaneous Multiplex Detection and Confirmation of the Proteinaceous Toxins Abrin, Ricin, Botulinum Toxins, and *Staphylococcus* Enterotoxins A, B, and C in Food

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Detection of proteinaceous toxins in complex heterogeneous mixtures requires highly specific and sensitive methods. Multiplex technology employing multiple antibodies that recognize different epitopes on a toxin provides built-in confirmatory analysis as part of the initial screen and thereby increases the reliability associated with both presumptive positive and negative results. Polyclonal and monoclonal antibodies were obtained for abrin, botulinum toxins, ricin, and *Staphylococcus* enterotoxins A, B, and C (SEA, SEB, and SEC). Food samples were spiked with the toxins either individually or mixed and analyzed following 40-fold dilution. Abrin, botulinum toxin A complex, ricin, and SEB displayed limits of detection in the original food samples ranging from 0.03 to 1.3 μg/mL, from 0.03 to 0.07 μg/mL, from 0.01 to 0.1 μg/mL, and from <0.01 to 0.03 μg/mL, respectively. Redundancy, that is, multiple antibodies for each toxin, some recognizing different epitopes or displaying different binding affinities, provided a "fingerprint" for the presence of the toxins and built-in confirmation, thus reducing the likelihood of false-positive and false-negative results. Inclusion of internal controls, including a unique protein, helped control for variations in dilution. Paramagnetic microspheres facilitated the detection of analyte in foods containing particulate matter incompatible with the use of filter plates normally used in the wash steps of assays employing standard polystyrene microspheres.

KEYWORDS: Abrin; botulinum toxin; ricin; *Staphylococcus* enterotoxin; food; multiplex; Luminex

INTRODUCTION

Numerous methods have been developed for the detection of proteinaceous toxins. However, antigen-specific assays for the screening of samples containing an unidentified toxin can be labor intensive, time-consuming, and expensive because multiple assays are required to screen for each toxin and to provide confirmatory analysis. Furthermore, a two-tier approach in which presumptive positives from an initial screen are subjected to a second confirmatory test to reduce false positives relies entirely on the initial analysis to prevent false negatives. Multiplex assays that simultaneously analyze a sample for multiple analytes can prevent these problems while also reducing potential sources of error associated with having to prepare multiple extracts of a sample for each analysis.

Various forms of multiplex assays have been developed, including microarrays (*1*) and bead-based assays (*2*−*5*), with detection based on fluorescence, electrochemiluminescence (ECL), Förster resonance energy transfer (FRET), fluorescence correlation spectroscopy, and catalyzed reporter deposition (CARD), among others (*4*, *6*−*9*). Although the detection of proteins using xMAP multianalyte profiling with fluorophore-encoded microspheres usually involves a sandwich design, competitive assays have been successfully applied to the detection of food adulterants such as soy and wheat food allergens (*10*). DNA-based detection using xMAP technology usually involves the detection of PCR-derived amplicons as exemplified by the detection of 23S rRNA amplicons for the foodborne pathogens *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, and *Campylobacter jejuni* (*11*) or padlock probe derived amplicons for pathogenic fungi (*12*). Other applications have included the detection in human sera of antibodies against polysaccharides and toxins of pathogens (*13*−*15*).

The inclusion of redundancy in multiplex assays, whereby multiple analyses target the same analyte, provides built-in confirmation and reduces the likelihood of false-positive and false-negative results. Furthermore, it is possible to characterize the sample in regard to unique structural properties by targeting these features as part of the redundancy. xMAP technology by Luminex relies on fluorophore-encoded microspheres to distinguish between up to 100 capture-based assays performed simultaneously on a single sample. By conjugating antibodies that recognize different epitopes and/or display different binding affinities to the same analyte to different fluorophore-coded microspheres, it is also possible to characterize the detected analyte relative to these

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properties. Multiplex assays ranging from 9- to 31-plex, which included up to 9 ricin, 2 abrin, 5 botulinum complex, 2 *Staphylococcus* enterotoxin A (SEA), 3 *Staphylococcus* enterotoxin B (SEB), 2 *Staphylococcus* enterotoxin C (SEC), and 8 control microsphere-based assays, were successfully employed to analyze food samples. The use of paramagnetic beads in place of nonmagnetic polystyrene beads facilitated the analysis of complex food matrices with a high content of particulate matter, such as chipotle mustard.

MATERIALS AND METHODS

Reagents. Botulinum toxin type A complex (supplied at 1 mg/mL, $MIPLD_{50}$ = 0.05 ng) and botulinum toxin type B complex (supplied at 1 mg/mL, $MIPLD_{50}$ = 0.03 ng) were purchased from Wako Chemicals (Richmond, VA). Botulinum toxin type E complex (supplied at 1 mg/mL, $MIPLD_{50}$ = 0.03 ng, trypsinized) was purchased from Metabiologics, Inc. (Madison, WI). Ricin RCA 60, ricin A-chain, ricin B-chain, and the agglutinin from castor beans (RCA 120) were purchased from Vector Laboratories (Burlingame, CA). Abrin fraction II was prepared for the FDA as previously described (*16*). *Staphylococcus* enterotoxins A, C1, C2, and C3 (SEA, SEC1, SEC2, and SEC3) were purchased from Toxin Technology, Inc. (Sarasota, FL). Streptavidin−*R*-phycoerythrin conjugate (SAPE) was purchased from Molecular Probes (Eugene, OR). *Staphylococcus* enterotoxin B and 10 mM PBS were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Fluorophore encoded MicroPlex microsphere (formerly named MultiAnalyte COOH microsphere) polystyrene beads and MagPlex microsphere superparamagnetic beads were purchased from Luminex Corp. (Austin, TX).

Antibodies. Rabbit and goat polyclonal antibodies and mouse monoclonal antibodies against the various toxins were the generous gifts of Tetracore, Inc., and the Biological Defense Research Directorate, U.S. Navy. The capture antibodies were conjugated to the polystyrene and paramagnetic beads according to the carbodiimide protocol (*17*). The detector antibodies, unless noted otherwise, were biotinylated using sulfosuccinimidyl-6-biotinamido-6-hexanamidohexanoate according to the manufacturer's instructions (Thermo Scientific Inc., Rockford, IL). Assays involving the use of monoclonal antibodies conjugated to microspheres and biotinylated polyclonal antibodies as the detector are indicated as mono/poly assays. Similarly, polyclonal antibodies conjugated to microspheres with biotinylated polyclonal antibodies as the detector are indicated as poly/poly assays.

Food Samples. Beverages (milk, soda, fruit juice, vegetable juices, infant formula, milk, and chocolate milk), condiments (Tabasco sauce and chipotle mustard), bread, and lettuce were purchased from a local grocery store. Food samples were prepared by spiking aliquots (v/v for beverages and w/v for solid food products) with freshly prepared solutions of abrin, botulinum toxin, ricin, and/or *Staphylococcus* enterotoxin in PBS. The spiked samples were incubated at room temperature for 30 min to 2 h prior to adding two internal controls in PBS, one a novel human protein and the second a hapten. The samples were then diluted 40-fold with 105 mM sodium phosphate/75 mM NaCl/2.5% nonfat dry milk (NFDM)/0.05% Tween 20 (UD buffer) prior to xMAP analysis using the Bio-Plex assays. UD buffer was used to prevent false positives associated with immunoassay analysis of samples containing lectins (*18*).

Bio-Plex Assay. Bio-Plex analyses consisted of 9 (9-plex) to 31 (31-plex) different microsphere assays performed simultaneously on a single sample. The Bio-Plex assays consisted of three incubation steps: mixing 100 μL of UD buffer diluted sample with 50 μL of a microsphere cocktail containing 4.5×10^4 of each capture antibody−fluorophore-encoded bead (30 min), mixing 50 μL of biotinylated detector antibody cocktail containing 0.25−0.5 μg of each detector antibody with the analyte−capture antibody−bead complex (20 min), and mixing 50 μL of 10 μg/mL SAPE in UD buffer with the biotinylated detector antibody−analyte−capture antibody−bead complex (5 min). The assay was performed at room temperature (21−23 °C) with the plate shielded from light and shaken at 750 rpm during each incubation step. Upon completion of the last incubation step, the bead complexes were suspended in 100 μL of PBS/0.1% Tween-20 (PBST) and transferred into polypropylene microtiter plates, and the median fluorescence intensity (MFI) of the beads was determined using a Bio-Plex 200 array reader. Assays involving the use of MicroPlex microsphere polystyrene beads were performed using MultiScreen-BV filter plates (Millipore Corp., Bedford, MA) and included three washings with PBST after each incubation step. When using MagPlex superparamagnetic microspheres, the microtiter plate was placed on a magnetic particle concentrator (Invitrogen Corp., Carlsbad, CA) for 5 min prior to decanting the liquid. A single wash step replaced the three washes normally performed after the first incubation step, the wash steps following the second and third incubations were eliminated, and only the solvent was decanted prior to the next step.

Microsphere bead sets conjugated to capture antibodies to detect the proteinaceous toxins and the internal standards (IS1 and IS2), along with a panel of Developmental AssayCheX bead sets (Radix Biosolutions, Georgetown, TX), were added as a cocktail of control microspheres to all samples. The Developmental AssayCheX bead sets monitor for addition of streptavidin phycoerythrin (fluorescence control, FC), addition of detector antibody (antibody control, AC), nonspecific binding (negative control, NC), and flow analyzer detection of fluorescence (instrument control, IC). Beads conjugated to antibodies against ovalbumin, *Yersinia pestis*, and *Francisella tularensis* were also added in some of the bead mixtures as controls for nonspecific binding. Biotinylated polyclonal antibodies were preferentially used as detector antibodies because these gave greater MFI values, presumably because multiple detector antibodies were able to bind a captured antigen, and facilitated the incorporation of redundant assays for the same antigen. The use of polyclonal antibodies as a common detector for multiple microspheres targeting the same antigen increases the likelihood that differences between the bead-based assays are due to the properties of the capture antibodies employed. This facilitates the development and application of detection profiles that can be used to characterize analytes.

xMAP multiplex assays were run on a Bio-Plex 200 array reader with Bio-Plex Manager 4.0 software (Bio-Rad Laboratories, Hercules, CA). The Bio-Plex was configured to count each sample for a maximum of 45 s with gates of 8500−18500 for the MicroPlex polystyrene beads and 7700−24700 for the MagPlex beads. The Bio-Plex 200 was calibrated using calibration and validation microspheres supplied by Bio-Rad Laboratories.

Data Analysis. Limits of detection (LOD) were calculated as the concentration of analyte necessary to generate a MFI >3 times the standard deviation above the background. Third-order polynomial fitting of the data (minimum of six concentrations, $R^2 \geq 0.99$) was used to calculate the LOD values. XLfit ver. 4.2 (ID Business Solutions Inc., IDBS, Alameda, CA) curve fitting software for Microsoft Excel was used to analyze the SEB concentration versus response profiles.

RESULTS AND DISCUSSION

Limit of Detection. Figure 1 compares the detection of abrin in PBS and in PBS/0.1% Tween-20/5% nonfat milk powder (PBSTM) using mouse monoclonal and rabbit polyclonal antibodies in different configurations within a 9-plex assay. As expected, use of the same monoclonal antibody for the capture and detector (mono/mono) resulted in no measurable response (data not shown). The LOD for abrin using monoclonal antibodies as the capture and polyclonal antibodies as the detector (mono/poly) averaged 0.7 ng/mL for PBS and PBSTM samples. This was slightly less than the 0.9 ng/mL average LOD for the opposite configuration (poly/mono) or the 11 ng/mL LOD for the poly/poly configuration. Assays employing rabbit polyclonal antibodies conjugated to the microspheres (poly/mono) displayed greater backgrounds than those using monoclonal antibodies (mono/poly). The background was further increased when biotinylated rabbit polyclonal antibodies were also used as the detector (poly/poly).

Changing the assay from a 9-plex to a 17-plex and incorporating the simultaneous detection of abrin, botulinum toxin, ricin, and SEB yielded the responses depicted in **Figure 2** and summarized in **Table 1**. The background responses for the various toxins varied from 60 to 400 MFI, depending on the assay, and were comparable to those measured in other multiplex assays. The 40-fold dilution of the samples with UD buffer resulted in comparable

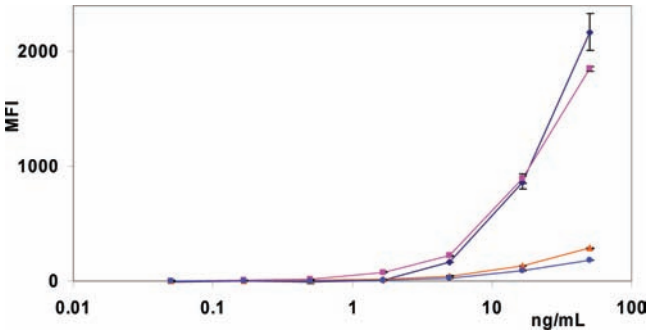

Figure 1. Detection of abrin. 9-plex assays containing mouse monoclonal antibodies against abrin conjugated to microspheres at 25 μg/mL, binding detected using biotinylated rabbit polyclonal antibodies (mono/poly), assayed in (black diamonds) PBS and (magenta squares) PBSTM, and rabbit polyclonal antibodies against abrin conjugated to microspheres at 125 μg/mL, binding detected using biotinylated mouse monoclonal antibodies (poly/mono), assayed in (orange triangles) PBS and (blue circles) PBSTM. The average responses of duplicate samples are plotted after subtraction of the background (110 $\pm$ 4 for mono/poly and 21 $\pm$ 2 for poly/mono) versus the concentration of abrin after mixing with the cocktail of microspheres. The error bars represent half the range between the responses generated by duplicate samples. Analyses employing rabbit polyclonal antibodies conjugated to microspheres with biotinylated rabbit polyclonal antibodies (poly/poly) generated results comparable to the poly/mono assay, but with a higher background of 285 $\pm$ 45.

results irrespective of whether the food matrix analyzed was chocolate milk, infant formula, vegetable juice, orange juice, diet cola, Tabasco sauce, baked bread (spiked after baking), lettuce (not all data shown), or PBS.

The LOD values for abrin detection from samples containing a mixture of abrin, botulinum toxin, ricin, and SEB using the mono/poly configuration ranged from 0.5 to 2 ng/mL (average = 1.4 ng/mL) for the 17-plex assay (**Table 1**). In contrast, the poly/poly configuration for abrin detection displayed an average LOD of 15 ng/mL. In both cases, the LOD values for the detection of abrin from a mixture of toxins were comparable to the LOD values measured using the 9-plex assay to detect abrin in PBS or PBSTM with no cross-reactivity to ricin. The same antibodies, when used in ELISA and electrochemiluminescent (ECL) assays, displayed LOD values of 0.1−0.5 ng/mL (*16*). Although the ELISA and ECL assays displayed lower LOD values, all three methods detected abrin at levels considerably less than the lethal dose based on an oral toxicity of > 1 mg/kg of bw (*19*) for a 10 kg person consuming 100 mL (or g) of a food product. Similarly, the LOD values for ricin using ricin-specific antibodies ranged from 10 to 100 ng/mL (0.1−2 ng/mL in the analytical sample), sufficient to detect an oral lethal dose of 20 mg/kg of bw (*19*, *20*). The LOD values for SEB using SEB-specific antibodies ranged from < 3 to 30 ng/mL, considerably less than a lethal dose based on the rhesus macaques nontoxic oral dose of 1 mg/kg of bw (*21*). The LOD for botulinum toxin A in food, using antibodies generated against botulinum toxins, varied from

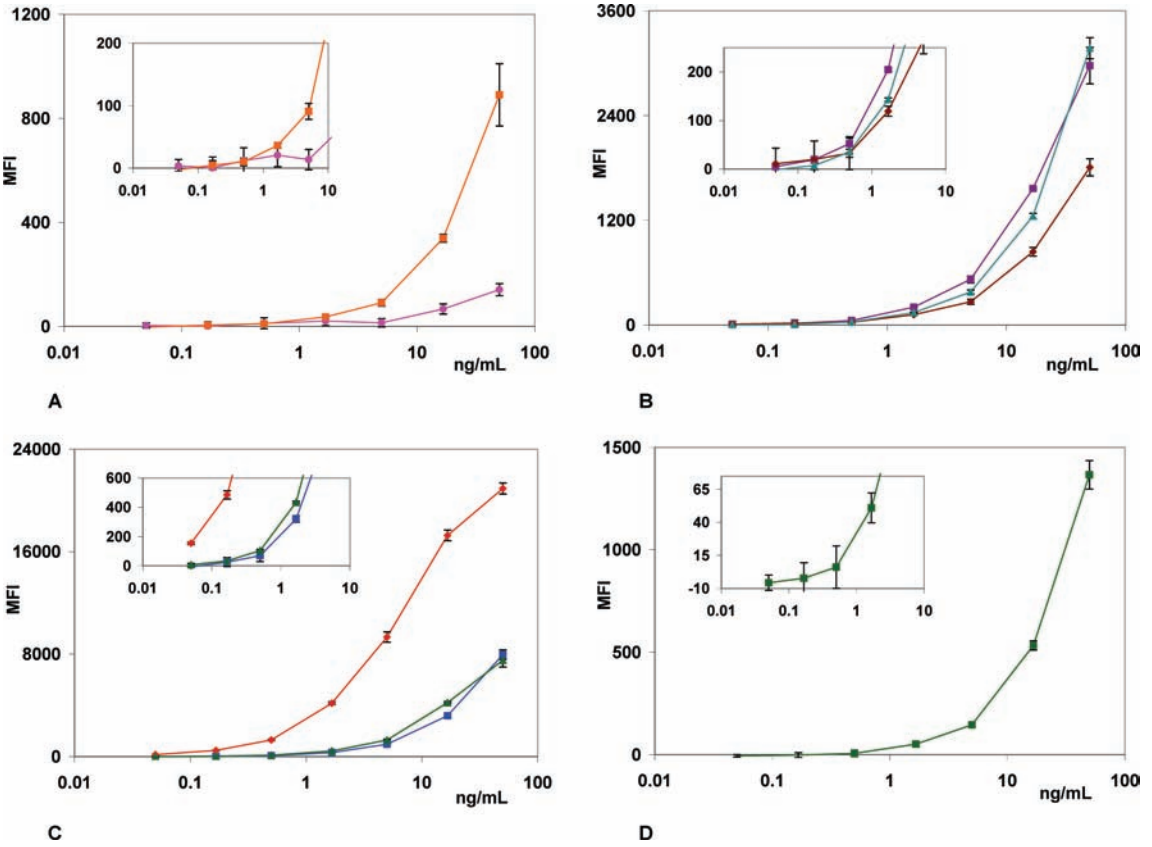

Figure 2. 17-plex analysis of chocolate whole milk spiked with abrin, botulinum toxin A, ricin, and SEB. Microsphere-based assays for the detection of (**A**) abrin using (orange squares) mouse mono/rabbit poly and (magenta circles) rabbit poly/rabbit poly; (**B**) ricin (violet squares) mouse mono/goat poly, (green triangles) goat poly/goat poly, and (brown diamonds) rabbit poly/goat poly; (**C**) SEB (green triangles) mouse mono-a/rabbit poly, (orange diamonds) mouse mono-b/rabbit poly, and (blue squares) rabbit poly/rabbit poly; and (**D**) botulinum toxin A (green squares) mouse mono/rabbit poly. Similar results were observed with PBS, vegetable juice, infant formula, orange juice, and diet cola. The average responses of triplicate samples are plotted versus the concentration of each proteinaceous toxin in the analytical sample following dilution and mixing with microspheres. Error bars represent the standard deviations.

Table 1. Simultaneous Detection of Proteinaceous Toxins[a]

	background[b]	LOD[c]		background	LOD		background	LOD	
	MFI ± SD	anal (ng/mL)	food (μg/mL)	MFI ± SD	anal (ng/mL)	food (μg/mL)	MFI ± SD	anal (ng/mL)	food (μg/mL)
	rabbit poly/rabbit poly[d]			mouse mono/rabbit poly					
abrin									
PBS	318 ± 17	14	0.8	162 ± 16	2.2	0.13			
chocolate milk	265 ± 17	11	0.7	63 ± 3	0.47	0.03			
infant formula	313 ± 22	12	0.7	160 ± 22	2.0	0.12			
vegetable juice	221 ± 12	22	1.3	111 ± 5	1.1	0.07			
	mouse mono/rabbit poly								
botulinum toxin A complex									
PBS	138 ± 7	1.2	0.07						
chocolate milk	98 ± 10	1.2	0.07						
infant formula	137 ± 17	1.0	0.06						
vegetable juice	97 ± 1	0.5	0.03						
	mouse mono/goat poly			goat poly/goat poly			rabbit poly/goat poly		
ricin									
PBS	134 ± 2	0.11	0.01	246 ± 5	0.19	0.01	200 ± 17	0.88	0.05
chocolate milk	67 ± 6	0.12	0.01	197 ± 34	1.7	0.10	83 ± 8	0.33	0.02
infant formula	126 ± 13	0.26	0.02	222 ± 12	0.35	0.02	207 ± 30	0.66	0.04
vegetable juice	157 ± 1	0.15	0.01	172 ± 4	0.54	0.03	210 ± 1	0.28	0.02
	mouse mono-a/rabbit poly			mouse mono-b/rabbit poly			rabbit poly/rabbit poly		
SEB									
PBS	296 ± 3	<[e]	<	404 ± 34	0.29	0.02	174 ± 6	0.20	0.012
chocolate milk	177 ± 13	<	<	235 ± 28	0.5	0.03	106 ± 7	0.088	0.005
infant formula	298 ± 18	<	<	395 ± 30	0.3	0.02	167 ± 12	0.18	0.011
vegetable juice	306 ± 15	<	<	265 ± 27	0.55	0.03	176 ± 7	0.26	0.016

[a] 17-Plex xMAP analysis. Spiked samples (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 μg/mL each toxin) were diluted 40-fold with UD buffer, then 100 μL mixed with 50 μL of polystyrene microsphere cocktail (4.5×10^4 of each bead set). [b] Background MFI ± standard deviation determined for triplicate analyses of samples spiked with 0 μg/mL of the proteinaceous toxin. [c] LOD values based on the concentration in the analytical sample after being diluted and mixed with microspheres (anal, ng/mL) and in the original food sample (μg/mL). [d] Antibody configuration of the assay: capture antibody conjugated to microspheres/biotinylated detector antibody. [e] Below range for reliable determination of an LOD value.

Table 2. Specificity of Polystyrene Microsphere xMAP Assays[a]

	bead	capture antibody	botulinum complex A	botulinum complex B	botulinum complex E
BOT	138	mAb[b]	100	11	1
	120	goat polyclonal	100	9	0
	121	goat polyclonal	100	8	0
	159	rabbit polyclonal	100	13	0
			ricin	**A-chain**	**B-chain**	**agglutinin**	**abrin**
ricin	122	mAb-a	100	0	45	16	0
	181	mAb-b	100	142	0	52	0
	182	mAb-c	100	1	27	21	0
	174	goat polyclonal	100	51	14	48	0
	149	rabbit polyclonal[c]	100	36	9	27	0
	175	rabbit polyclonal[c]	100	35	9	27	0
			SEA	**SEB**	**SEC1**	**SEC2**	**SEC3**
SEA	179	rabbit polyclonal	100	72	9	6	0
SEB	124	mAb-a	15	100	7	0	0
	155	mAb-b	46	100	32	0	0
	133	rabbit polyclonal	33	100	32	3	3
SEC	177	mAb	1	0	100	43	26
	176	rabbit polyclonal	2	53	100	40	40

[a] MFI responses normalized relative to the immunogen used to generate the antibodies except for the botulinum antibodies, which were normalized relative to the response of botulinum complex type A. A single biotinylated polyclonal antibody was used as the detector antibody for each toxin. [b] Mouse monoclonal antibodies, mAb-a, mAb-b, ..., indicate different monoclonal antibodies. [c] The same rabbit antiricin polyclonal antibody was conjugated to microspheres 149 and 175.

30 to 70 ng/mL (0.5−1.2 ng/mL in the analytical sample). This is sufficiently sensitive to detect the lethal dose for a 10 kg person consuming 100 g of adulterated food based on an estimated oral lethal dose of 1 μg/kg of bw (*22*−*24*).

Table 3. $y = (A \exp(-B/x))$ Analysis of SEB Assays[a]

microsphere conjugate	sample	*A* coefficient	*B* coefficient	R^2
SEB[b]				
SEB-124	PBSTM	17600	57	1.00
SEB-155	PBSTM	24000	13	0.99
SEB-133	PBSTM	15900	43	1.00
mixture of proteinaceous toxins [c]				
SEB-124	PBSTM	17400	46	1.00
	orange juice	16400	49	1.00
	vegetable juice	16300	55	1.00
	diet cola	17100	48	1.00
	av ± SD	16800 ± 500	50 ± 4	
SEB-155	PBSTM	25000	10	0.99
	orange juice	24500	10	0.99
	vegetable juice	25200	12	0.99
	diet cola	25200	10	0.99
	av ± SD	25000 ± 300	11 ± 1	
SEB-133	PBSTM	13700	32	1.00
	orange juice	13600	33	1.00
	vegetable juice	13600	38	1.00
	diet cola	13900	34	1.00
	av ± SD	13700 ± 100	34 ± 3	
recalibration prior to analysis [c,d]				
SEB-124	PBS	9400	29	0.99
	chocolate milk	12100	32	0.99
	vegetable juice	11200	34	0.99
	infant formula	10700	27	0.98
	av ± SD	11000 ± 1000	31 ± 3	
SEB-155	PBS	20100	5.9	0.99
	chocolate milk	21800	5.5	0.99
	vegetable juice	21800	5.5	0.99
	infant formula	20100	4.3	0.99
	av ± SD	21000 ± 1000	5.3 ± 0.7	
SEB-133	PBS	9020	22	0.98
	chocolate milk	9300	18	0.99
	vegetable juice	10600	24	0.99
	infant formula	9270	17	0.98
	av ± SD	9500 ± 700	20 ± 3	

[a] Dose−response curve of samples spiked with 0, 0.003, 0.01, 0..03, 0.1, 0.3, 1, and 3 μg/mL SEB fit to $y = A \exp(-B/x)$, where x is concentration (ng/mL) after 40-fold dilution with UD buffer and y is MFI generated by 100 μL of UD diluted samples mixed with 50 μL beads. Samples run in triplicate. MFI values corrected for background. R^2 = correlation coefficient. [b] SEB was the only select agent spiked into the samples. [c] Samples also spiked with abrin, botulinum toxin, and ricin and analyzed in a 17-plex assay. [d] BioPlex recalibrated prior to analysis.

Internal Standards. To facilitate the analysis of complex samples and provide a gauge for whether the food or its handling might affect the magnitude of the MFI, two internal standards were incorporated into all samples. One of the internal standards (IS1) was a novel human protein that was assayed for using polyclonal antibodies as both the capture (conjugated to microspheres) and the detector (biotinylated). The detection of IS1 required all of the steps associated with detecting the proteinaceous toxins. The other internal standard (IS2) was configured to circumvent the need for biotinylated detector antibodies. The MFI values of IS1 and IS2 in the samples used to generate the data in **Figure 2** ranged from 530 to 620 and from 830 to 910, respectively, with average %CV values of ≤5%. The lack of significant differences in MFI values indicates that a correction was not necessary due to loss or mishandling. Similar invariance was observed with the Developmental AssayCheX bead sets (FC, NC, AC, and IC), which displayed average MFI values of 3000, 60, 50, and 1370 with average %CV values of 10, 6, 4, and 2%, respectively.

Specificity. No cross-reactivity of >2% of the background MFI was observed between the assays for the ribosome-inhibiting proteins (RIPs) abrin and ricin with botulinum toxin or *Staphylococcus* enterotoxin at concentrations in the analytical sample ranging up to 0.33 μg/mL, equivalent to 20 μg/g of food. Similarly, the *Staphylococcus* entertoxin assays did not display cross-reactivities of >2% of the background MFI with botulinum toxins or RIPs at similar concentrations. Furthermore, those assays that employed monoclonal antibodies conjugated to the microspheres displayed cross-reactivities of ≤1% of the background MFI. Higher levels of cross-reactivity, ranging from 10 to 25%, were observed between the botulinum assays and *Staphylococcus* enterotoxins that did not correlate with either an elevated background or whether polyclonal or monoclonal antibodies were conjugated to the microspheres.

The cross-reactivities of several assays with related proteins and subunits are presented in **Table 2**. The responses presented in **Table 2** were normalized relative to the MFI generated by the immunogen used to generate the capture antibodies, except for the botulinum toxin assays that were normalized relative to the MFI generated by botulinum complex type A. By incorporating multiple assays that target different epitopes that represent different features of the antigen in a single analysis, it is possible to generate a fingerprint that increases the reliability of the analysis and characterizes the analyte. This approach is exemplified by the ricin and *Staphylococcus* enterotoxin assays. By incorporating the various ricin microspheres into an analysis, it is possible to determine whether the sample contains individual ricin subunits (i.e., ricin A- or B-chains), the holoprotein, or abrin. Similarly, inclusion of *Staphylococcus* enterotoxin specific microspheres 124, 133, 155, 176, 177, and 179 makes it possible to detect and distinguish between SEA, SEB, and SEC. Determination of whether the SEC present is SEC1, SEC2, or SEC3 is not possible without ascertaining through an independent method the concentration of *Staphylococcus* enterotoxin present and quantifying the specific activity. In addition, by conjugating the same capture antibody onto multiple microspheres, an additional level of built-in confirmation is achieved as illustrated by beads 149 and 175, which employ the same rabbit polyclonal anti-ricin capture antibodies and generated similar results.

Kinetic Profile. Table 3 compares the coefficients necessary to fit the detection profiles for the three SEB assays to the exponential expression $y = A \exp(-B/x)$, where x represents the concentration of SEB and y the observed MFI. The ratios between the coefficients for the three SEB assays (SEB-124:SEB-155:SEB-133) used to detect SEB alone or among other proteinaceous toxins were comparable at 1.1:1.5:1 and 1.2:1.8:1 for the A coefficients and 8.6:2:6.5 and 9.1:2:6.2 for the B coefficients. Recalibrating the Bio-Plex changed the magnitude of the coefficients, although the ratios for the A and B coefficients were only slightly altered to 1.2:2.2:1 and 11.6:2:7.5, respectively. The observation that similar results were obtained irrespective of the beverage spiked with SEB illustrates advantages of diluting the samples 40-fold with UD buffer prior to analysis. The extensive dilution minimizes sample carry-over, whereas the NFDM and detergent in UD buffer make the environmental milieu sufficiently complex that it overwhelms any carry-over and facilitates the dissolution of the analyte from beverage components. Hence, the A and B coefficients are virtually constant. Thus, whenever the three SEB assays are employed, the expected ratios between the coefficients should be observed. This provides an additional level of confirmation in addition to an increase in MFI.

Magnetic Microspheres. The high concentration of particulate matter of various densities in chipotle mustard make it unsuitable for analysis using polystyrene microspheres that require filter plates for removal of reagents in the wash steps. **Figure 3** depicts the results obtained by analyzing chipotle mustard spiked with

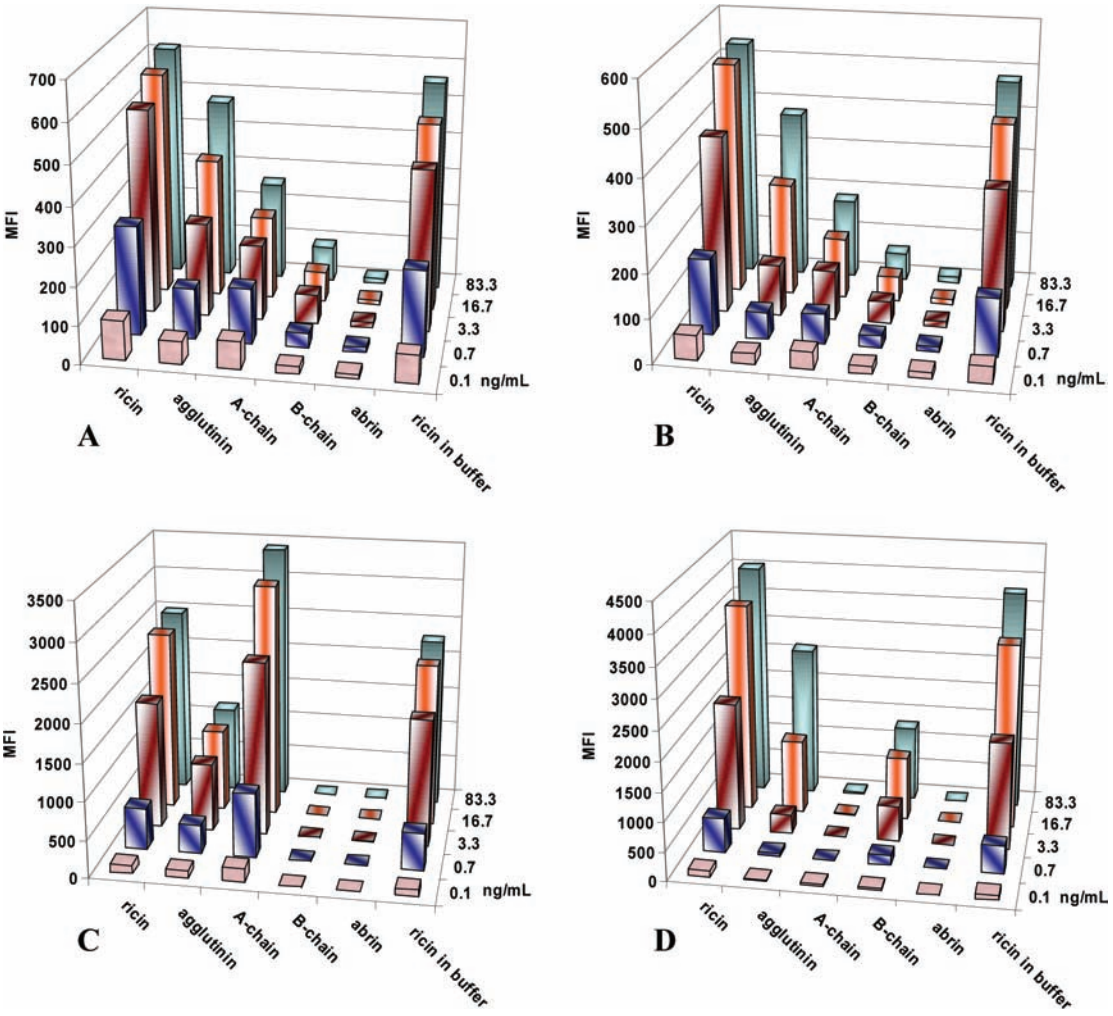

Figure 3. Ricin detection in mustard using MagPlex microspheres. Ricin, A-chain, B-chain, agglutinin from *Ricinus communis*, and abrin spiked in chipotle mustard were assayed using (**A**) goat anti-ricin polyclonal, (**B**) rabbit anti-ricin polyclonal, (**C**) A-chain specific mouse monoclonal, and (**D**) B-chain specific mouse monoclonal antibodies conjugated to MagPlex superparamagnetic microspheres. Biotinylated rabbit anti-ricin polyclonal antibodies were used to detect binding. Ricin spiked into UD buffer was used as a control. The average %CV values for the capture antibodies ranged from 6 to 12%.

either ricin, ricin A-chain, ricin B-chain, agglutinin from *R. communis*, or abrin using ricin antibodies conjugated to MagPlex superparamagnetic microspheres. The antibodies displayed the expected A-chain and B-chain specificities. The observation that larger MFI values were observed with capture monoclonal antibodies and not polyclonal antibodies is consistent with prior observations favoring mono/poly configurations. Of further use, the antibodies displayed differences in affinity (**Figure 4**) that can be used as described previously for SEB detection.

The use of magnetic microspheres provided a useful alternative to polystyrene microspheres by enabling the analysis of samples containing particulate matter that would otherwise prevent the use of the filter plates routinely used with polystyrene beads while not requiring either centrifugation or filtration. Furthermore, 40-fold dilution with UD buffer generated a sample compatible with LFD, ELISA, and ECL analysis (*17*), thereby providing additional sources of confirmation should such be needed.

A recently published study on the detection of similar proteinaceous toxins reported lower LOD values with best sensitivity observed upon minimizing the number of capture beads, increasing the sample volume, and increasing the incubation time to 16 h to maximize the amount of analyte per bead (*25*). Although this approach effectively increased sensitivity, a balance between sensitivity, throughput/time of analysis, and advantages associated with built-in confirmation is also important. Inasmuch as the focus is protection from intoxication via ingestion, the assays must be able to reliably detect less than the oral lethal dose. Another study examined the detection of ricin and SEB in powdered drinks and powdered food products (*26*). However, this study required centrifugation and filtration of the dissolved samples prior to analysis, did not include built-in confirmation, and displayed reduced sensitivity with milk (108 ng/mL ricin in the analytical sample). Minimizing sample preparation is important for analytical methods in which automation is important either to maximize throughput or for methods used infrequently and not routinely subjected to proficiency testing. Dilution minimizes both analytical labor and the effects of sample matrix variability on the performance of the assay.

xMAP multiplex technology enabled the simultaneous analysis of a single sample for abrin, botulinum toxin, ricin, and *Staphylococcus* enterotoxins in food samples with limits of detection sufficient to detect less than an oral lethal dose. The use of redundancy, whereby multiple assays are simultaneously performed for the same analyte that target the same or different structural features, provides built-in confirmation that reduces the likelihood of both false-positive and false-negative results and can be used to characterize analytes. The compromise between multiple detection, redundancy, sensitivity, minimizing sample

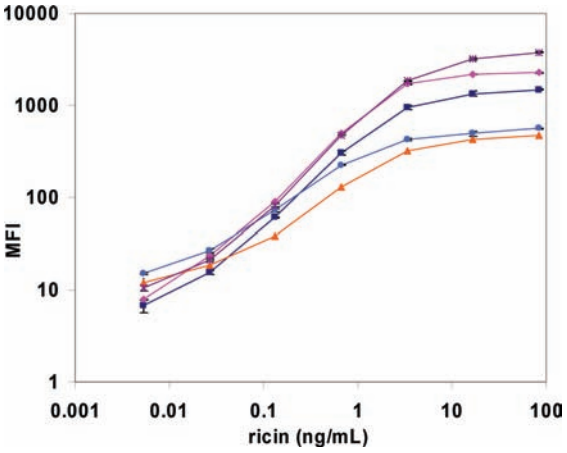

Figure 4. Ricin detection using MagPlex microspheres: concentration versus MFI profiles for the detection of ricin in UD buffer using (blue circles) goat polyclonal, (orange triangles) rabbit polyclonal, (magenta diamonds) A-chain specific, and (black squares and brown asterisks) two B-chain specific mouse monoclonal antibodies as the capture antibodies conjugated to the MagPlex microspheres. The average MFI responses of samples prepared in triplicate are plotted versus the concentration of analyte in the analytical sample. The error bars represent the standard deviations.

preparation, and generating confirmatory data resulted in LOD values from comparable to approximately 10-fold greater than some protein specific assays and biosensors. However, as long as the LOD values are less than an oral lethal dose, it is not necessarily desirable that all assays for a specific toxin display comparably low LOD values. Assays with various sensitivities and binding affinities display different kinetic properties that provide an additional level of confirmation and characterization. Ultimately, the ability to distinguish the presence of different proteinaceous toxins or identify multiple toxins present in a single sample depends on the degree of cross-reactivity or the uniqueness (stringency) of the assays employed and the degree of redundancy in assaying different features.

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