



Published in final edited form as:

J Agric Food Chem. 2013 January 23; 61(3): 755–760. doi:10.1021/jf3041963.

Detection of Botulinum Neurotoxin Serotypes A and B Using a Chemiluminescent versus Electrochemiluminescent Immunoassay in Food and Serum

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Abstract

Botulinum neurotoxins (BoNT) are some of the most potent biological toxins. High-affinity monoclonal antibodies (mAbs) have been developed for the detection of BoNT serotypes A and B using a chemiluminescent capture ELISA. In an effort to improve toxin detection levels in complex matrices such as food and sera, we evaluated the performance of existing anti-toxin mAbs using a new electrochemiluminescence (ECL) immunoassay developed by Meso Scale Diagnostic instrument. In side-by-side comparisons, the limit of detection (LOD) observed for ELISA and the ECL immunoassay for BoNT/A was 12 pg/mL and 3 pg/mL, and for BoNT/B was 17 pg/mL and 13 pg/mL, respectively. Both the ELISA and the ECL method were more sensitive than the “gold standard” mouse bioassay. The ECL assay outperformed ELISA in detection sensitivity in most of the food matrices fortified with BoNT/A, and in some food spiked with BoNT/B. Both the ELISA and the ECL immunoassay platforms are fast, simple alternatives for use in the routine detection of BoNTs in food and animal sera.

Keywords

Botulinum neurotoxin; electrochemiluminescence; ELISA; immunoassay; monoclonal antibodies; mouse bioassay; food matrix; serum

INTRODUCTION

Botulinum neurotoxins (BoNTs), the causative agent of botulism, are some of the most lethal human bacterial toxins.¹⁻³ Intentional food or environmental contamination using BoNTs is a bioterrorism concern and thus they are classified as Select Agents by both the Centers for Disease Control and Prevention and the U.S. Department of Agriculture. While there are seven BoNT serotypes (A-G), serotypes A, B and E, cause most of the human botulism cases classified as infant, wound or food-borne botulism.¹ The only treatments available for botulism remain the use of respiratory aids (ventilators) and the neutralization of excess toxin in the bloodstream with an equine derived polyvalent anti-toxin serum or in the case of infant botulism, with the human derived antitoxin BabyBig.⁴ The speed of recovery depends on quick diagnosis and treatment.

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SAFETY Botulinum neurotoxins are Select Agents and extremely toxic. Special handling requirements, with appropriate PPE and use of appropriate biological safety cabinets are required.

Many BoNT detection assay platforms have been described over the years with some reporting sensitivities at the attomolar level.⁵⁻⁸ However, most are not designed for detection of BoNT in complex matrices such as food, biological, or environmental samples. The current “gold standard” for detection of BoNTs remains the mouse bioassay, one of the most sensitive and robust methods.^{9,10} The mouse bioassay measures BoNT in minimal lethal dose (MLD) units, which is the lowest dose at which all tested mice die. Mice are monitored over several days for signs of intoxication and death.^{11,12} Toxin serotype is identified in a subsequent mouse bioassay incorporating an additional antibody protection step using serotype specific anti-toxin antibodies. Sensitivity of the mouse bioassay is in the range of 20-30 pg/mL for BoNT/A and 10-20 pg/mL for BoNT/B.^{13,14} The mouse bioassay is slow needing at least four days to complete, requires the use live animals, and uses death as an endpoint.

Recently, *in vitro* detection methods such as traditional ELISA have been developed in our laboratory for BoNT/A and BoNT/B.^{15,16} These assays, based on mAbs, have detection sensitivities lower than the mouse bioassay. Using a new electrochemiluminescence (ECL) immunoassay platform, we described a method for detecting BoNT/A in mouse sera with a sensitivity of 10 pg/mL.¹⁷ The ECL platform uses an immunoassay format much like an ELISA but the output signal is not produced by enzymatic hydrolyses of a luminescent substrate. Instead, a luminescent signal is generated by an electron cycling of the Ruthinium label. In the experiments described here a Sector Imager 2400 from Meso Scale Discovery (MSD) was used. ECL microplates contain carbon electrode surfaces and used ruthenium labelled (SULFO-TAG™) anti-toxin mAbs that emit light only when brought into close proximity of the electrodes coated with a different capture mAb. This format reduces background light emission and matrix effects. In this study, we compared the detection sensitivities for BoNT/A and BoNT/B in different liquids, liquefied solid foods, and horse serum by the ELISA and the ECL assay. Direct comparison of the performance of different assays in a variety of complex matrices provides important information useful for determining antibody performance on different platforms and for choosing a format before more extensive inter-laboratory validation studies.

MATERIALS AND METHODS

Toxins and antibodies

BoNT/A and BoNT/B holotoxins and rabbit polyclonal antibodies against BoNT/A and BoNT/B were purchased from Metabionics (Madison, WI). Toxin was diluted in phosphate gelatin buffer (0.01 M phosphate buffer pH 6.2 and 2% gelatin), aliquoted, and frozen at -80 °C. Fresh aliquots were used for each experiment. Monoclonal antibodies (mAbs) for BoNT/A: F1-2, F1-40, F2-43, and F1-51 were described previously.^{16,18,19} Anti-BoNT/B mAb MCS 6-27 was described previously¹⁶ and mAb BoB92-32 is a newly developed antibody from our laboratory (unpublished results).

Mouse bioassays

Groups of at least 10 randomly sorted female Swiss Webster mice (19-22 g) were used. Mice were housed in groups of 5 in standard animal room conditions with unlimited access to food and water. Mice were dosed with 0.5 mL of 3 pg to 100 pg per mouse (Table 1) of BoNT/A or BoNT/B holotoxin by the intraperitoneal (ip) route. Mice were monitored for botulism symptoms (wasp waist, difficulty with breathing, paralysis, etc.) for up to 8 days post-intoxication. The mean lethal dose was calculated by the Reed and Muench method.²⁰ Median survival for each dose was calculated using GraphPad Prism 5 (San Diego, CA). The mouse bioassays were performed according to animal-use protocols approved by the Animal Use and Care Committee of the USDA.

Sample preparation

Toxin standards were made in TBS-T-NFM (20 mM Tris-HCl buffer, pH 7.4, 0.9 % NaCl; 0.05% Tween-20, and 3 % non-fat dry milk powder). Apple, orange, and carrot juices, non-fat milk, whole milk, as well as carrot, pea, and sweet potato puree were purchased from a local grocery store. Apple and orange juices were neutralized with the addition of 5 M Tris pH.8 (10 % final volume) before the addition of toxin. Orange juice was centrifuged briefly (500 X g for 2 min) to remove large solids. All samples were spiked with equal volumes of toxin previously diluted to working concentrations in their respective matrices (Tables 2-4). Liquid samples were prepared by adding 10 μ L diluted toxin (640, 160, 40, 10, 2.5, 0.62, 0.16 ng/mL) to 1 mL of liquid matrix. Pureed foods were prepared by two methods: (a) with no dilution - by adding 100 μ L of 1.1 X working toxin solution (70.4, 17.6, 4.4, 1.1, 0.27, 0.069, 0.017 ng/mL) to 1 g of pureed food; or (b) with buffer dilution - by adding 10 μ L toxin to 0.5 g of pureed food followed by the addition of 500 μ L PBT buffer (PBS with 1% BSA and 0.05% Tween-20). Pureed food samples were then centrifuged at 13,000 X g for 5 min. 600 μ L supernatant was collected and used for detection assays. Toxin preparations were diluted in buffer, food and serum matrices and used for mouse bioassays, ELISA or ECL detection.

Electrochemiluminescence assays

MA2400 96-Well standard ECL plates (MSD, Gaithersburg, MD) were treated with 30 μ L/well of a 2 μ g/mL solution of anti-BoNT/A specific mAb F1-2 or with anti-BoNT/B specific mAb MCS-6-27 in PBS and 0.05 M carbonate-bicarbonate buffer pH 9.6, respectively. These antibodies function as the capture reagent for both assay formats. Plates were then blocked with 200 μ L/well of TBS-T-NFM for 1 h with shaking at 37 °C. Next, -0 μ L/well of toxin standards in TBS-T-NFM as well as spiked food and serum samples, were added and the microassay plates incubated at 37 °C with shaking for 1 h. Plates were then washed 3X with TBS-T buffer. For BoNT/A plates, 30 μ L/well of a 3 μ g/mL antibody solution (1 μ g/mL ea. of biotinylated F1-51, F1-40, and F2-43) was added to wells. For the BoNT/B plates, 30 μ L/well of a 2 μ g/mL solution of biotinylated BoB92-32 was added to the wells. The plates were then incubated at 37 °C for 1 h, washed 3 X in TBS-T. For the ECL assay, 30 μ L/well of a 1:1000 dilution of a ruthenium-conjugated SULFO-TAG™ Streptavidin (MSD) was added and the plates incubated for 1 h at 37 °C with shaking. Plates were then washed 3X with TBS-T buffer and 200 μ L/well of 1X Read Buffer T with surfactant (MSD) was added before reading with a Sector Imager 2400 (MSD). The amount of toxin present in food and sera matrices was determined by comparison to the toxin standards in TBS-T-NFM included on each plate. Recovery percentage of BoNTs in food matrices was determined by comparing the unknown signal to that of the buffer standard using the MSD Discovery Workbench software program. Each plate contained standards and samples in duplicate wells. Relative standard deviation (RSD %) was determined from 3 to 6 independent assays. For ease of comparison, toxin standard curves for the ECL assay were plotted using a non-linear regression curve fit with second order polynomials, and the statistical significance of the ECL assay and the ELISA LODs were determined using the Prism 5 program. LOD was determined by the addition of background signal plus three standard deviations and determining the concentrations from the X-axis in the graph. The LODs were reported as mean plus the standard error of the mean (SEM, n = 4). Results from typical standard curves for BoNT/A and BoNT/B are shown in Figure 1A.

ELISA assays

The capture ELISA used here was previously described^{15,16} but was modified as follows. Serotype A specific capture mAb F1-2 or serotype B specific mAb MCS6-27 (2 μ g/mL) in carbonate buffer was absorbed on the surface of microtiter wells. Plates were blocked with TBS-T-NFM. Toxin standards were added to TBS-T-NFM or food matrices and the plate

incubated at 37 °C for 1 h followed by 6X wash with TBS-T. For detection of BoNT/A, a mixture of biotin-labeled mAbs F1-40, F1-51, and F2-43, each at 1 µg/mL, was added and the plates incubated at 37 °C for 1 h. For detection of BoNT/B, 100µL/well of a 1µg/mL solution of biotin-labeled mAb BoB92-32 was added. The plates were then washed as above. Streptavidin-HRP (0.1 µg/mL, Sigma) was added and the plates incubated at 37 °C for 1 h. The plates were then washed 9X as above and the luminescent substrate SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) was added and incubated for 3 min at room temperature with gentle agitation. Luminescent counts were recorded using a Wallac Victor 3 Multilabel Counter (PerkinElmer Inc., Waltham, MA). Standards and samples were added in duplicates. Relative standard deviation (RSD %) was determined from 3 to 6 independent assays. Recovery percentage of BoNTs in food matrices was determined by comparing the unknown signal to that of buffer standard using GraphPad Prism 5. Standard curves for BoNT/A and BoNT/B were plotted and the LODs calculated as described above for ECLs. Results from typical standard curves are shown in Figure 1B.

RESULTS AND DISCUSSION

Mouse bioassay

The mouse bioassay is currently used as the “gold standard” for the detection of BoNTs. While sensitive, the method is subject to large variations and ambiguous results. Toxin detection is defined in minimum lethal dose (MLD) units, that is the dose resulting in death of both mice in a group of two. However, animal bioassay outcomes are often dependent on the type of animals used and test conditions, making the reproducibility of results difficult in different laboratories. The results from a mouse bioassay of a larger animal sampling size ($n = 10$) per toxin dose are indicated in Table 1. Results observed at intermediate ranges of lethality, such as with 25 and 12.5 pg/mL for BoNT/A (6 deaths out of 10 and 14 deaths out of 20 mice, respectively) and 12.5 pg/mL for BoNT/B (6 deaths out of 10 mice), make the determination of a consistent MLD difficult. The larger the sample size, the more accurate the results, but also the larger the number of animals needed with subsequent ethical implications. The detection limits (based on MLD) determined using the mouse bioassay, with the same toxin batch used in the immunoassays, were 50 pg/mL and 25 pg/mL for BoNT/A and BoNT/B, respectively.

Assay optimization

Before comparing the detection sensitivities of ELISA and the ECL assay in food, we tested different buffer and antibody combinations to determine the optimum assay conditions. We tested the use of single mAb, multiple mAbs and rabbit polyclonal antibodies for the detection of BoNT/A in capture type immunoassays. However, we opted to design our assays with monoclonal antibodies because commercial polyclonal antibodies were expensive and not of unlimited quantities and consistent quality. For detection of BoNT/A in either the ELISA or the ECL assay, mAb F1-2 was used as the capture antibody. We obtained the best detection sensitivities in either the ECL or ELISA platforms using rabbit polyclonal antibodies as detector antibodies (data not shown). To simulate the effect of polyclonal antibodies, we tested the use of a mixture of mAbs as detector antibodies. A combination (1:1:1) of biotinylated mAbs F1-40, F1-41 and F2-43 each at 1 µg/mL gave the best detection sensitivities for BoNT/A compared to single or a sets of two mAbs.¹⁹ For BoNT/B detection, mAb MCS6-27 was used as the capture mAb and biotinylated mAb BoB92-32 was used the detector mAb. Only a single mAb was available for BoNT/B detection at this time. Neither sets of mAbs against BoNT/A and BoNT/B cross-reacted with other BoNT serotypes.^{15-16,18,21}

Using the optimized assay, the limit of detection (LOD) for the ELISA assay in TBS-T-NFM buffer matrix was 12 ± 2 pg/mL for BoNT/A and 17 ± 6 pg/mL for BoNT/B, (Figure 1B); the LOD for the ECL assay was 3 ± 0.4 pg/mL and 13 ± 4 pg/mL for BoNT/A and BoNT/B, respectively (Figure 1A). The LOD was calculated by determining mean assay activity for the zero toxin concentration (the background signal) plus three standard deviations. The LOD difference of ELISA vs. ECL assays for BoNT/A, although small, was statistically significant (p value of 0.0003 by a two-tailed unpaired t -test). There is no significant difference in LOD for BoNT/B detection between the two methods.

Detection and recovery of BoNTs in complex food matrices

We tested the detection of BoNT/A and BoNT/B in non-fat and whole milk, and in apple, orange and carrot juices. Liquid matrices required very little processing. Both the apple and orange juices had low pH values <3 that interfere with antibody-antigen interactions. Therefore, we neutralized these low pH juices with a 5 M Tris buffer (pH 8). The detection sensitivities for BoNT/A in liquid matrices (Table 2) are denoted as recovery percentages for a known spiked level of toxin in the sample. The ECL assay detected toxin in all of the liquid matrices with a better sensitivity than observed in the ELISA. Using the ECL method, detection in the 2-6 pg/mL ranges for most liquid matrices was observed while the ELISA platform detected toxin in the 6-25 pg/mL range.

Pureed food matrices represent a more complex matrix than juices and toxin detection presented additional challenges. Low recovery yields were observed when carrot and sweet potato puree were analyzed using either the ELISA and ECL platforms. The ECL assay outperformed the ELISA in sensitivity and recovery percentage (Table 3). These results demonstrate a matrix effect from liquefied solid foods. In the case of carrots, matrix effect was observed in puree but not in juice (Table 2). To lessen these matrix effects, we diluted food samples 1:1 with PBT buffer (PBS with 1% BSA and 0.05% Tween-20) followed by centrifugation to remove the particulate material. Results using the diluted supernatants are shown in Table 3. Clearly, dilution improved recovery dramatically for the carrot and sweet potato matrices. However, BoNT/A detection in the diluted pea puree using the ECL platform was unsatisfactory. For the pea puree the dilution buffer led to an unusually high signal for all spike levels less than 6400 pg/mL making calculation of recovery unreliable. It is unclear why there was more interference, perhaps a higher BSA concentration in samples was not compatible with the ECL format.

Recovery of BoNT/B in all of these same liquid matrices was slightly better with the ECL method than with the ELISA platform (Table 4). However, detection sensitivity for carrot and pea puree was slightly better with the ELISA platform than with the ECL platform after buffer dilution. We also compared the detection sensitivities of BoNTs in horse serum and found no significant difference in detection sensitivities and toxin recoveries for BoNT/A or BoNT/B in either immunoassay (Tables 2 and 4).

Both the ELISA and the ECL immunoassay platforms detected toxins at levels below that detected with the mouse bioassay and in considerable shorter times (5 h for immunoassays vs. 4-8 days for the mouse bioassay). The immunoassays used less sample per test, an average of 30 μ L for the ECL, 100 μ L for ELISA vs. 500 μ L for the mouse bioassay, a consideration when limited quantities of clinical samples are available. Our studies highlighted differences in assay performance, e.g., sensitivity between different assay platforms even when the same mAbs are used. Clearly, different antibodies and matrices can affect assay performance, but the platform used also can influence performance even when the same antibodies are used (e.g., compare toxin detection in the pea puree with the ECL vs. the ELISA). Matrix pH also plays an important role on immunoassays as low pH conditions affect antibody-antigen interactions. Routine buffering of samples should be

considered when designing assays and different buffering agents should be optimized for each method as some buffers may interfere with individual platforms. However, simple dilution of a complex matrix can greatly improve detection sensitivity. Overall, detection of BoNTs was better in the ECL platform than with the ELISA platform in most of the food matrices we tested. In addition, the ECL assay platform is amenable to multiplexing, allowing serotype identification simultaneous with toxin detection. The ECL platform carries higher costs than that for the ELISA because of more expensive assay plates and instrumentation. However, the benefits of the smaller sample size, lower reagent use, and the potential for multiplexing could offset the cost disadvantages.

This study is the first to directly compare an ELISA method with the new MSD ECL assay platform in different food and biological matrices using the same samples and antibody reagents. Previous studies compared ELISA with a paramagnetic bead based ECL assay (BioVeris analyzer) using the same monoclonal antibodies and determined detection thresholds in different sample matrices ranging from 0.78–1.56 ng/mL²² as well as the detection sensitivity of a commercial kit with the BioVeris based ECL (50 pg/mL and 100 pg/mL for BoNT/A and BoNT/B, respectively).²³ The results presented here profile new assays adding to the arsenal of detection platforms evaluated for BoNT detection. The detection sensitivities presented here for the ELISA and the ECL assay should prove sufficient for the detection of toxin levels found in real life samples.⁶

A major advantage of the mouse bioassay is that it can detect active toxin, while immunoassays usually cannot distinguish active vs. non-active toxin. The mouse bioassay can also detect multiple BoNT serotypes and subtypes, while immunoassays are dependent on the quality of mAbs used and will only detect those serotypes and subtypes recognized by the antibodies incorporated into the test. Clearly antibodies used in an immunoassay need to be tested for binding using as many toxin serotypes and subtypes as are available. However, because of the much shorter time required, immunoassays represent good candidates for preliminary tests and could be used to provide rapid diagnosis on large sample populations and significantly reduce the number of animals used for confirmation.

Acknowledgments

We thank Thomas D. Henderson II, Wanless Hatcher and Zeke Martinez for technical and animal facilities support; James Lindsay, and Kirkwood Land for their helpful comments. Also, we thank Meso Scale Discovery for the loan of the Sector Imager 2400 instrument. The authors were funded by the United States Department of Agriculture, Agriculture Research Service, CRIS project 5325-42000-048-00D, the National Institute of Allergy And Infectious Diseases Service Grant U54 AI065-59, and DHS agreement 40768.

ABBREVIATIONS USED

BoNT	botulinum neurotoxin
ECL	electrochemiluminescence
ELISA	enzyme-linked immunoabsorbent assay
mAbs	monoclonal antibodies
MLD	minimal lethal dose
LOD	limit of detection
MSD	Meso Scale Discovery
ip	intraperitoneal
SEM	standard error of the mean

(RSD) relative standard deviation

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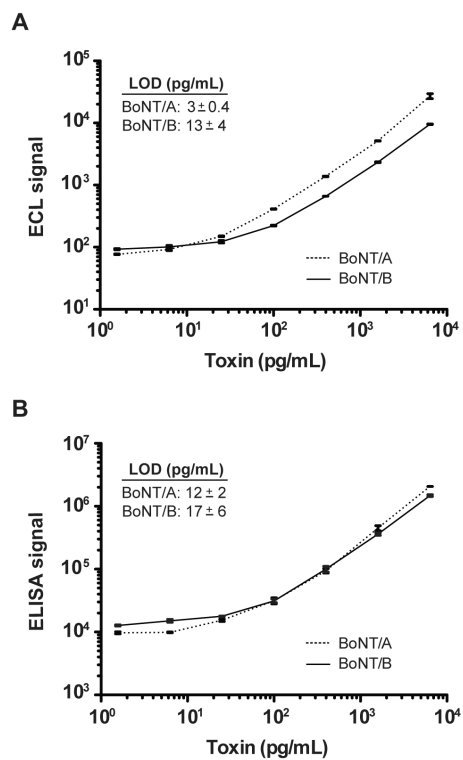


Figure 1. Standard curves for detection of BoNT/A and BoNT/B. A. ECL assay. B. ELISA. Curves for BoNT/A and BoNT/B were obtained in TBS-T containing 3% non-fat dry milk. The limit of detection (LOD) was calculated as background signal plus three standard deviations. The LOD value shown represents the average \pm standard error of the mean (SEM) with $n = 4$.

Table 1

Mouse Bioassays of Mice Treated with Different Doses of BoNT (Holotoxin) Serotypes A and B by ip Injections.

Toxin	Dose (pg/mL)	LD ₅₀ Unit ^a	# Dead/Total ^b	Median Survival (h) ^c
BoNT/A	100	10	15/15	< 24
	50	5	16/20	< 24
	25	2.5	6/10	26.5
	12.5	1.3	14/20	53.5
	6.25	0.64	0/10	--
	3.12	0.32	0/10	--
BoNT/B	50	4	9/10	< 24
	25	2	9/10	39
	12.5	1	6/10	63
	6.25	0.5	0/10	--
	3.12	0.25	0/10	--

^aUnits were based on mouse intraperitoneal (ip) LD₅₀. Estimated BoNT/A holotoxin ip LD₅₀ was 9.8 pg/mouse or 0.44 ng/kg and LD₅₀ for BoNT/B holotoxin was 12.5 pg/mouse or 0.55 ng/kg

^bSurvival is denoted as number of dead mice over total number tested.

^cComparison of survival curves for different doses using the Log-rank Test showed $p = < 0.0001$

Table 2

Percentage of Recovery for BoNT/A in Liquid Food Matrices

Initial BoNT/A concentration (pg/mL) ^a							
Samples	6400	1600	400	100	25	6.2	1.6
Whole Milk	73 (27)	79 (26)	80 (28)	72 (34)	69 (24)	75 (38)	79 (36)
Non-Fat Milk	78 (29)	82 (22)	82 (22)	90 (25)	77 (10)	81 (8)	83 (6)
Apple Juice	46 (50)	45 (48)	46 (49)	60 (39)	86 (23)	--*	--
Orange Juice	51 (27)	58 (21)	57 (29)	65 (28)	74 (24)	61 (46)	--
Carrot Juice	88 (19)	85 (24)	88 (25)	80 (21)	82 (25)	101 (9)	42 (83)
Horse Sera	73 (10)	76 (2)	79 (6)	89 (9)	112 (11)	--	--

Initial BoNT/A concentration (pg/mL) ^b							
Samples	6400	1600	400	100	25	6.2	1.6
Whole Milk	68 (13)	71 (20)	73 (23)	73 (35)	67 (14)	86 (64)	--
Non-Fat Milk	71 (10)	80 (20)	78 (22)	76 (21)	78 (41)	73 (21)	--
Apple Juice	39 (57)	30 (77)	20 (12)	22 (71)	--	--	--
Orange Juice	45 (32)	40 (47)	34 (59)	34 (62)	46 (39)	88 (41)	--
Carrot Juice	83 (22)	95 (13)	96 (17)	65 (27)	54 (81)	--	--
Horse Sera	96 (10)	86 (9)	78 (8)	63 (8)	41 (42)	--	--

^aDetection by ECL. Percent recovery: average (RSD %)

* Not reliably detected

^bDetection by ELISA. Percent recovery: average (RSD %)

Table 3

Percentage of Recovery for BoNT/A in Pureed Food Matrices

Initial BoNT/A concentration (pg/mL) ^a								
Method	Samples	6400	1600	400	100	25	6.2	1.6
ECL	Carrot	10 (3)	12 (8)	11 (10)	14 (9)	12 (12)	22 (23)	21 (71)
	Pea	101 (22)	85 (7)	82 (4)	90 (14)	109 (5)	--	--
	Sweet Potato	13 (49)	14 (24)	12 (9)	12 (34)	12 (13)	6 (61)	--
ELISA	Carrot	4 (34)	4 (24)	2 (102)	--	22 (72)	--	--
	Pea	71 (25)	71 (14)	64 (17)	52 (10)	41 (81)	--	--
	Sweet Potato	8 (24)	8 (20)	6 (18)	10 (27)	--	--	--
Initial BoNT/A concentration (pg/mL) ^b								
Method	Samples	6400	1600	400	100	25	6.2	1.6
ECL	Carrot	101 (5)	105 (8)	107 (7)	116 (14)	--	--	--
	Pea	112 (3)	--	--	--	--	--	--
	Sweet Potato	65 (13)	72 (7)	73 (9)	78 (12)	82 (32)	85 (31)	--
ELISA	Carrot	83 (4)	86 (3)	81 (8)	72 (4)	59 (61)	--	--
	Pea	102 (4)	111 (7)	106 (13)	86 (42)	--	--	--
	Sweet Potato	59 (16)	67 (26)	60 (36)	46 (43)	17 (89)	--	--

^aRecovery of pureed foods without dilution. Percent recovery: average (RSD %)^bRecovery of pureed foods after 1:1 buffer dilution. Percent recovery: average (RSD %)

Table 4

Percentage of Recovery for BoNT/B in Food Matrices.

Initial BoNT/B concentration (pg/mL) ^a							
Samples	6400	1600	400	100	25	6.2	1.6
Whole Milk	84 (2)	85 (2)	88 (3)	92 (1)	92 (18)	56 (45)	--
Non-Fat Milk	81 (4)	83 (3)	88 (4)	95 (4)	100 (9)	91 (35)	--
Apple Juice	53 (3)	52 (2)	58 (3)	88 (7)	--	--	--
Orange Juice	69 (6)	65 (9)	70 (8)	68 (18)	71 (21)	28 (5)	9 (101)
Carrot Juice	76 (6)	75 (6)	78 (8)	74 (13)	78 (17)	64 (74)	48 (53)
Carrot Puree	97 (2)	88 (2)	100 (9)	102 (26)	--	--	--
Pea Puree	95 (9)	98 (5)	103 (7)	113 (10)	--	--	--
Sweet Potato Puree	81 (18)	78 (7)	75 (5)	88 (2)	103 (18)	--	--
Horse Sera	70 (8)	72 (11)	85 (14)	108 (2)	--	--	--

Initial BoNT/B concentration (pg/mL) ^b							
Samples	6400	1600	400	100	25	6.2	1.6
Whole Milk	102 (3)	102 (7)	102 (9)	99 (18)	109 (14)	117 (1)	--
Non-Fat Milk	104 (3)	104 (5)	104 (13)	95 (8)	81 (21)	--	--
Apple Juice	66 (7)	52 (9)	40 (19)	27 (11)	--	--	--
Orange Juice	73 (15)	64 (9)	55 (5)	42 (17)	--	--	--
Carrot Juice	100 (8)	94 (7)	87 (10)	74 (20)	57 (51)	93 (7)	--
Carrot Puree	90 (2)	82 (6)	83 (9)	66 (32)	33 (28)	--	--
Pea Puree	93 (5)	96 (3)	92 (8)	73 (7)	45 (38)	--	--
Sweet Potato Puree	84 (11)	71 (13)	61 (8)	51 (17)	--	--	--
Horse Sera	88 (4)	79 (5)	88 (11)	107 (12)	--	--	--

^aDetection by ECL. Percent recovery: average (RSD %)^bDetection by ELISA. Percent recovery: average (RSD %)