



Detection of Botulinum Toxins A, B, E, and F in Foods by Endopep-MS

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ABSTRACT: Botulism is caused by exposure to botulinum neurotoxins (BoNTs). BoNTs are proteins secreted by some species of clostridia; these neurotoxins are known to interfere with nerve impulse transmission, thus causing paralysis. Botulism may be contracted through consumption of food either naturally or intentionally contaminated with BoNT. The human lethal dose of BoNT is not known but is estimated to be between 0.1 and 70 μ g; thus, it is important to be able to detect small amounts of this toxin in foods to ensure food safety and to identify the source of an outbreak. Our laboratory previously reported on the development of Endopep-MS, a mass-spectrometric-based endopeptidase method for the detection and differentiation of BoNT. This method can detect BoNT at levels below the historic standard mouse bioassay in clinical samples such as serum, stool, and culture supernatants. We have now expanded this assay to detect BoNT in over 50 foods including representative products that were involved in actual botulism investigations. The foods tested by the Endopep-MS included those with various acidities, viscosities, and fat levels. Dairy and culturally diverse products were also included. This work demonstrates that the Endopep-MS method can be used to detect BoNT/A, /B, /E, and /F in foods at levels spiked below that of the limit of detection of the mouse bioassay. Furthermore, we successfully applied this method to investigate several foods associated with botulism outbreaks.

KEYWORDS: botulism, Endopep-MS, botulinum toxins, mass spectrometry

INTRODUCTION

Botulinum neurotoxin (BoNT) is generated by *Clostridium botulinum* in addition to some other clostridia species; exposure to the toxin causes botulism, a potentially fatal neuromuscular disease. Botulism can be contracted through consumption of food containing BoNT as certain clostridia grow well in an anaerobic environment, such as improperly stored or preserved foods.^{1,2} Structurally, the neurotoxin is a dichain toxin, comprised of a light chain which has enzymatic activity and a heavy chain responsible for receptor binding and delivery of the light chain to its target. The enzymatic activity of the light chain is responsible for the toxicity of BoNTs as the light chain of BoNT cleaves proteins necessary for the release of the neurotransmitter, acetylcholine; the lack of acetylcholine causes flaccid paralysis.^{3–13}

Although the case–fatality ratio of foodborne botulism has decreased dramatically over the last few decades, foodborne botulism continues to be problematic due to rapid food modernization. During the period from 1990 to 2000, for instance, there were 263 cases of foodborne botulism reported in the United States.¹⁴ There are seven confirmed serotypes of BoNT, A–G, and four of those serotypes, BoNT/A, /B, /E, and /F have been reported to cause foodborne botulism; serotypes E and A are the most common in the United States.¹⁴ To date, there have been no known human foodborne botulism cases caused by serotypes C, D, or G. Foodborne botulism can

be prevented by preserving foods through established practices such as high heat to reduce spores and/or use of additives, such as salt or sugar, to prevent toxin production and by storing minimally processed food at the required temperature and for no longer than recommended.¹

There are a number of published in vitro techniques such as immunoassays for BoNT detection in foods.^{15–17} Our laboratory has previously described the development of the Endopep-MS method to detect BoNTs present in buffer.¹⁸ This method is an in vitro activity assay and detects the enzymatic action of the toxin light chain on a peptide substrate which mimics the toxin's in vivo protein target. Cleavage of the peptide substrate is monitored by mass spectrometry such that the exact location of the substrate cleavage is determined by examining the mass of the cleavage products. Since the substrate cleavage location is serotype specific, the various BoNTs can be differentiated by serotype. The use of an immunoaffinity step prior to incubation with the peptide substrate has proven effective at detecting and differentiating BoNT in clinical specimens¹⁹ and culture supernatants.²⁰ The

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Table 1. Peptide Sequences for the Endopep-MS Method Listed Along with the Observed ($M + H$)⁺ of Substrate and Cleavage Products for Each Serotype^a

peptide	sequence	<i>m/z</i> observed
SubA	Acetyl-RGSNPKIDAGNQ ⁺ RATRLGGR-NH ₂	2406
SubA NT product	Acetyl-RGSNPKIDAGNQ	1427
SubA CT product	RATRLGGR-NH ₂	999
SubB	LSELDDRADALQAGASQFESSAAKLKRKYWWKNLK	4025
SubB NT product	LSELDDRADALQAGASQ	1760
SubB CT product	FESSAAKLKRKYWWKNLK	2283
SubE	WWWAKLGQ ⁺ EIDTRNRQK ⁺ DhRIMAKADSNKR-NH ₂	3614
SubE NT product	WWWAKLGQ ⁺ EIDTRNRQK ⁺ DhR	2501
SubE CT product	IMAKADSNKR-NH ₂	1133
SubF	TSNRRLQQTQAQVDEVVDIMRVNVDKVL ⁺ ERDQ ⁺ KLSELDDRADAL	5111
SubF NT product	TSNRRLQQTQAQVDEVVDIMRVNVDKVL ⁺ ERDQ	3784
SubF CT product	KLSELDDRADAL	1346

^aX represents norleucine, and hR represents homoarginine.

Endopep-MS method achieves limits of detection comparable to or below that of the historically used mouse bioassay.²¹

On the basis of our previous success in detecting and differentiating BoNT in clinical specimens and culture supernatants, we sought to extend the utility of the assay to foods. In this work, we used the Endopep-MS method to detect BoNT/A, /B, /E, and /F spiked into more than 50 foods. In all cases, BoNT/A, /B, /E, and /F were detectable at levels spiked below the limit of detection of the mouse bioassay. For 10 diverse types of foods, the limit of detection was determined. Many of the foods evaluated were submitted during suspected botulism outbreaks over the past several years, and the foods account for a wide variety of characteristics such as pH, viscosity, and fat content and are representative of cuisines from varied ethnic cultures.

MATERIALS AND METHODS

Materials. Monoclonal antibodies to BoNT/A (CR2 and RAZ1),²² BoNT/B (2B18.2 and B12.1),²³ BoNT/E (4E17.1),²⁴ and BoNT/F (6F5)²⁵ were obtained from Dr. James Marks at the University of California at San Francisco and used for immunoaffinity purification. Dynabeads (M-280/Streptavidin) were purchased from Invitrogen (Carlsbad, CA). BoNT/A (3.5×10^7 mLD₅₀/mg), BoNT/B (1.1×10^7 mLD₅₀/mg), BoNT/E (3.0×10^7 mLD₅₀/mg), and BoNT/F (3.6×10^6 mLD₅₀/mg) complex toxins at a concentration of 1 mg/mL, where mLD₅₀ is the mouse lethal dose, were purchased from Metabio (Madison, WI). Botulinum neurotoxin is very toxic and necessitates suitable safety measures (see below). All chemicals were from Sigma-Aldrich (St. Louis, MO) except where indicated. Peptide substrates for evaluation of BoNT activity are listed in Table 1 and were synthesized by Midwest Biotech Inc. (Fishers, IN). Sulfo-NHS-Biotin was purchased from Thermo Fisher Scientific (Waltham, MA). Kingfisher plates (deep well and traditional 96 well) and tip combs were purchased from Thermo Fisher Scientific (Waltham, MA).

Preparation of Food Extracts. Foods were purchased from local stores. For the spiking studies, extracts were made from solid foods prior to analysis. The solid food was weighed and placed into a standard 80 mL stomacher bag. A 1 mL amount of phosphate-buffered saline with 0.05% tween-20 (PBST) was added for every gram of food, with a minimum of 10 g and a maximum of 60 g of processed. Foods were processed using the Stomacher 80 Biomaster (Seward, Port Saint Lucie, FL) at maximum speed for 2 min. The mixture was then centrifuged at 12 000g in a refrigerated centrifuge (4 °C) for 20 min. The supernatant was recovered, and centrifugation continued until a clear supernatant was obtained. The clear supernatant was used for testing. Liquid foods were not processed prior to use. BoNT was

pipetted into the clear supernatant or liquid food in volumes varying from 2 to 20 μ L.

For foods from outbreak investigations, 5–10 g (or 5–10 mL if liquid) of sample was transferred to a container and 1 mL of gelatin phosphate-buffered collecting fluid (GBS) per 1 g (or 1 mL) of sample was added. After mixing, the sample was centrifuged at 4 °C for 20 min at 23 000 g. The supernatant was then transferred into a 6 or 10 mL sterile syringe and filtered through a 0.45 μ m filter.

Preparation of mAb-Coated Beads. The mAbs (20 μ g) were biotinylated with 4 μ L of the 300 μ M sulfo-NHS-biotin in water, prepared immediately before use. The mAb/biotin was incubated overnight at room temperature with no mixing. Biotin-labeled mAb (2 μ g) was added to 100 μ L of washed (two times with 1 mL of PBST; resuspended in 100 μ L of PBST) streptavidin-coated beads and incubated at room temperature for 1 h with constant movement to keep the beads in solution. The beads then were washed twice in 1 mL each of PBST, and then the mAb bound beads were reconstituted in 100 μ L of PBST. For a higher number of test samples, the volumes were increased proportionally.

Spiking Liquid Foods and Food Extracts with BoNT. Biosafety Level-2 practices, processes, and facilities were used to ensure safety while working with BoNT. Additionally, toxin stock material and all samples containing BoNT were processed in a Class II biosafety cabinet containing HEPA filters to minimize the potential for aerosol exposure.

An aliquot of the liquid food or food extract of 0.5 or 1 mL was placed into a deep well plate. BoNT/A, /B, /E, or /F complexes were serially diluted in PBST from 1000 mLD₅₀/μL down to 0.001 mLD₅₀/μL immediately prior to spiking using 2 μ L of the stock solution and 18 μ L of PBST. For milk, the following levels were spiked: 10 mLD₅₀, 1 mLD₅₀, 0.5 mLD₅₀, 0.25 mLD₅₀, 0.1 mLD₅₀, 0.05 mLD₅₀, and 0.01 mLD₅₀ of BoNT. For infant formula, yogurt, broccoli, green bean liquid, salami, salmon, liquid egg, orange juice, and tomato juice, the following levels were spiked: 1 mLD₅₀, 0.75 mLD₅₀, 0.5 mLD₅₀, 0.25 mLD₅₀, 0.1 mLD₅₀, 0.05 mLD₅₀, 0.025 mLD₅₀, and 0.01 mLD₅₀ of BoNT. The remaining foods were spiked with 0.75 mLD₅₀, 0.5 mLD₅₀, or 0.25 mLD₅₀ of BoNT. The limit of detection was defined as the lowest level of toxin which could be detected during five separate analyses performed by a minimum of two analysts. Detection of the toxin consisted of the presence of mass spectrometric peaks with S/N of greater than 3 times above the S/N of the negative control (unspiked sample of same matrix).

Extraction of BoNT from Foods. For spiked foods, 10X PBST was added to achieve a final volume of 0.55 (for 0.5 mL of food extracts) or 1.1 mL (for 1 mL of food extracts). All spiked foods were tested in parallel with the same volume of blank food matrix, diluted to the same level with 10X PBST. For foods from outbreak investigations, an aliquot of 100 μ L was placed into a deep well plate along with 400 μ L of 1X PBST. A negative control consisting of 100 μ L of blank food matrix and 400 μ L of 1X PBST and a positive control consisting of 100

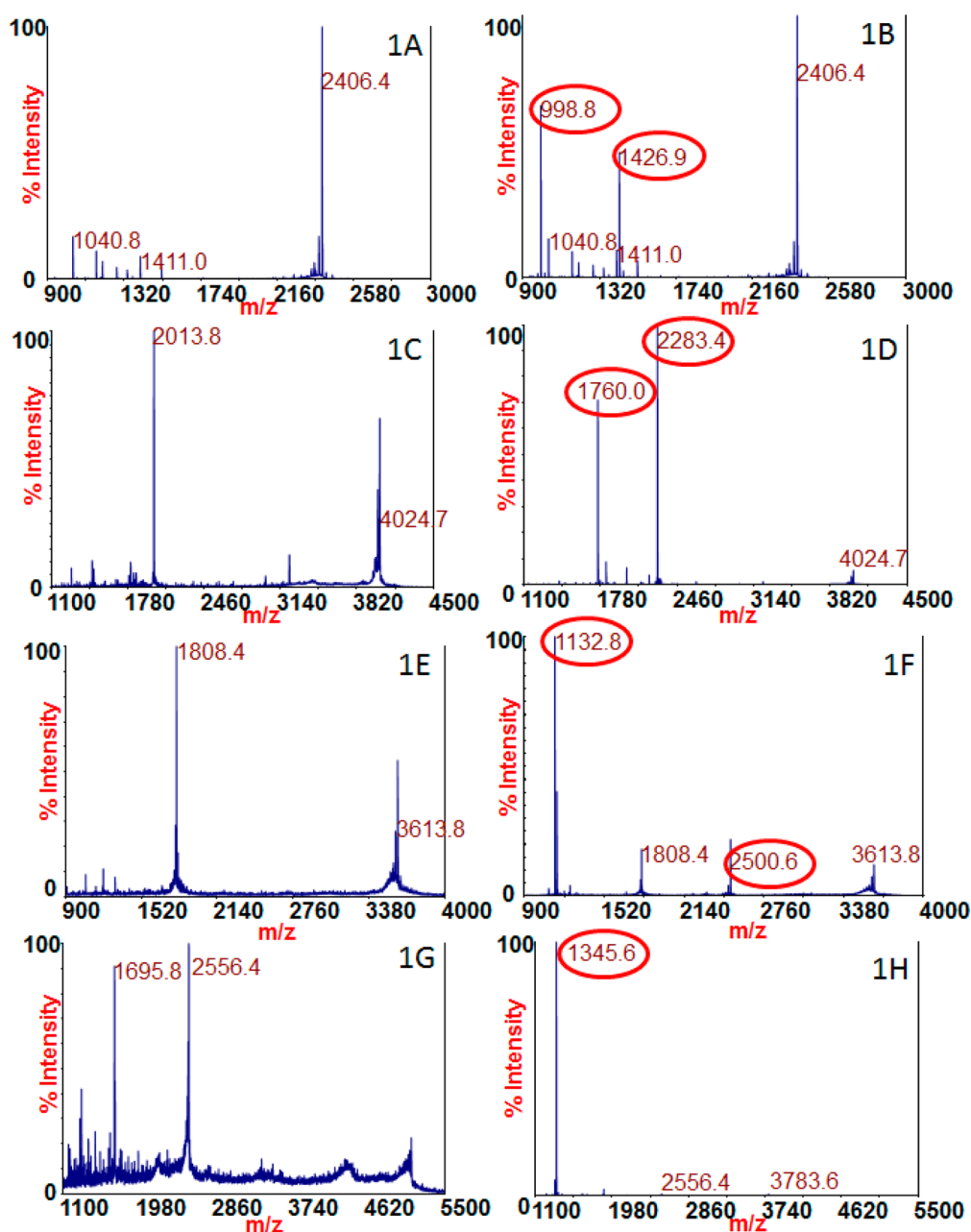


Figure 1. Mass spectra of 10 mLD₅₀ of (B) BoNT/A, (D) BoNT/B, (F) BoNT/E, and (H) BoNT/F spiked into 1 mL of milk. Cleavage products indicating the presence of BoNT are circled in red. Negative controls consisted of 1 mL of milk tested for (A) BoNT/A, (C) BoNT/B, (E) BoNT/E, and (G) BoNT/F.

μ L of blank food matrix, 400 μ L of 1X PBST, and 1 mLD₅₀ of BoNT were run in parallel with the food from the outbreak investigation. The foods from outbreak investigations included salmon, kim chi, sofrito, salsa, rice, juice, steak, pebre, corn cake, chili sauce, tomatoes, tomato paste, tofu, soup, fish, sauerkraut, relish, tea grounds, sour cream dip, yogurt, rice, vegemite, chili peppers, and baked potato. In addition, acidic matrices were adjusted to a pH of 7.0 with the addition of ammonium hydroxide unless otherwise noted.

Antibody-coated beads (20 μ L) were added to the sample. The deep well plate was capped and placed on a plate shaker for 1 h at the minimal speed necessary to keep the beads in solution. The deep well plate was then uncapped and placed into a KingFisher flex magnetic particle processor (Thermo Fisher Scientific, Waltham, MA) for automated bead washing, which included two washes with 1 mL each of 2 M NaCl (or 1X PBST as specified) followed by two washes with 1 mL each of 1X PBST. The beads were eluted into 80 μ L of water and

removed from the KingFisher flex. Replicate extracts were incubated with each serotype of mAb coated beads.

Endopep-MS Reaction with MALDI-TOF Analysis. The aqueous extract was removed from the beads, and then the beads were reconstituted in 18 μ L of reaction buffer consisting of 0.05 M Hepes (pH 7.3), 25 mM dithiothreitol, and 20 μ M ZnCl₂ and 2 μ L of peptide substrate specific for the antibody extract (SubA, SubB, SubE, or SubF, respectively). The final concentration of each substrate was 50 pmol/ μ L. All samples were incubated at 37 °C for 4 h with no agitation. A 2 μ L aliquot of each reaction supernatant was mixed with 18 μ L of matrix solution consisting of α -cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA), and 1 mM ammonium citrate. A 0.5 μ L aliquot of this mixture was pipetted onto one spot of a 384-spot matrix-assisted laser desorption/ionization (MALDI) plate (Applied Biosystems, Framingham, MA). Mass spectra of each spot were obtained by scanning from m/z 900 to 5500 in MS-positive ion reflector mode on an Applied

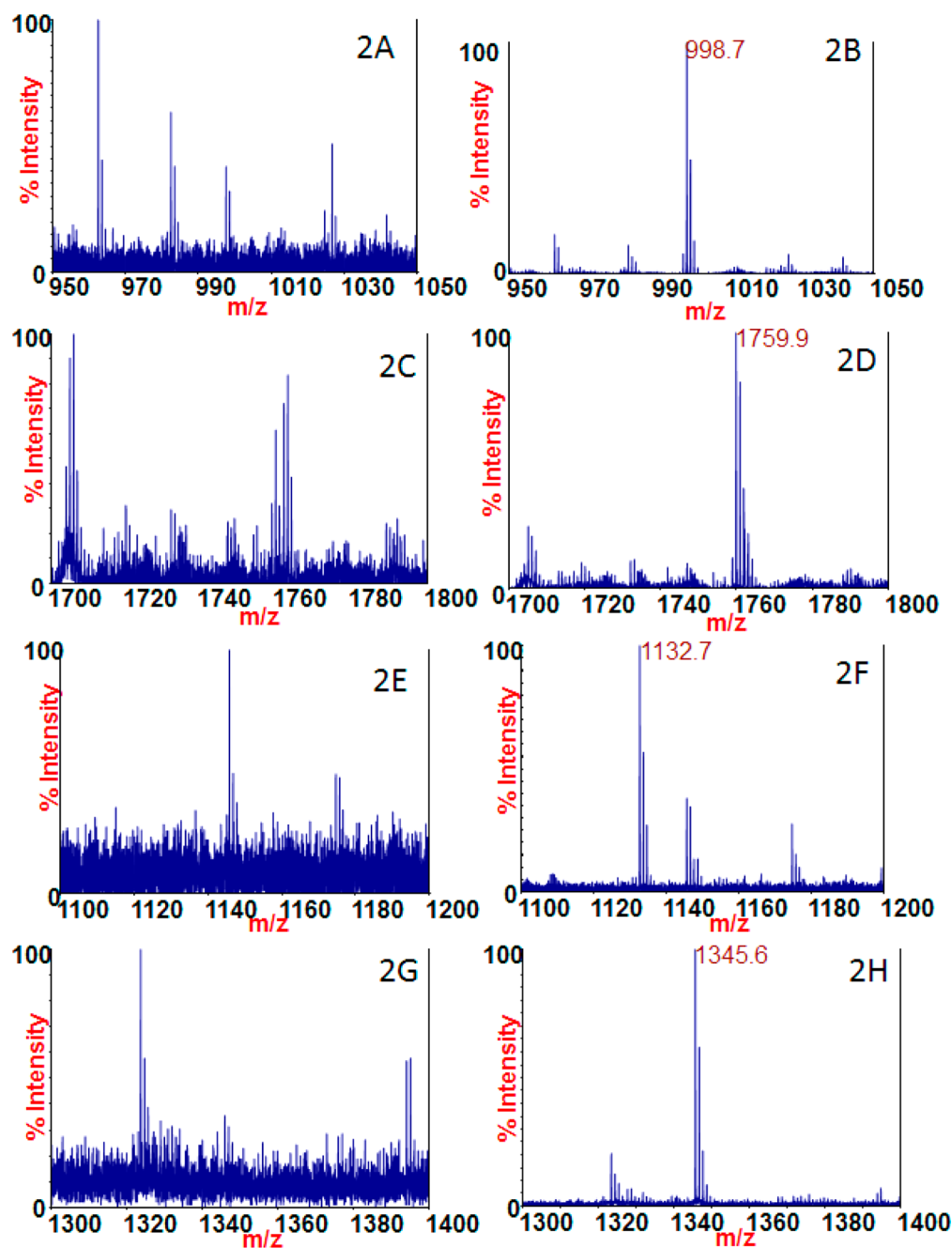


Figure 2. Mass spectra of (B) 0.25 mL_{D₅₀} of BoNT/A, (D) 0.1 mL_{D₅₀} of BoNT/B, (F) 0.25 mL_{D₅₀} of BoNT/E, and (H) 0.01 mL_{D₅₀} of BoNT/F spiked into 1 mL of milk. Negative controls consisted of 1 mL of milk tested for (A) BoNT/A, (C) BoNT/B, (E) BoNT/E, and (G) BoNT/F. Spectra are zoomed to display the dominant cleavage product.

Biosystems 5800 Proteomics Analyzer (Framingham, MA). The instrument uses an Nd:YAG laser at 355 nm, and each spectrum is an average of 2400 laser shots.

RESULTS AND DISCUSSION

Detection of BoNT/A, /B, /E, and /F in Whole Milk. The Endopep-MS method has been used successfully in the analysis of clinical specimens and culture supernatants for BoNT/A, /B, /E, and /F, demonstrating its potential to diagnose botulism, a potentially fatal disease. Although this method has limits of detection in clinical specimens and culture supernatants lower than that of the historically standard assay, the mouse bioassay, until now it was uncertain if the assay could be adapted to examine foods directly for the presence of BoNT/A, /B, /E,

and /F. Detection of BoNT in foods is important as it allows for discovery of the vehicle of transmission in foodborne botulism cases, potentially preventing additional outbreaks. Foods represent a wider variety of different characteristics than clinical specimens. For example, foods can be protease rich, low pH, high fat, and very viscous, all properties which could negatively affect any assay for detection of BoNT. We therefore wanted to test the Endopep-MS method on a wide variety of foods with varying properties in addition to foods which have been known to serve as causative agents of botulism in the past.

We began with the analysis of BoNT/A in whole milk. Various levels of BoNT/A were spiked into whole milk and analyzed. Comparison of the mass spectra of whole milk

Table 2. Limit of Detection of BoNT/A, /B, /E, and /F Spiked into 10 Selected Food Matrices

food	food state	BoNT/A	BoNT/B	BoNT/E	BoNT/F
milk	liquid	0.25 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL
infant formula	liquid	0.5 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL
green bean liquid	liquid	0.5 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL
orange juice ^a	liquid	0.25 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.5 mLD ₅₀ /mL	0.025 mLD ₅₀ /mL
tomato juice ^a	liquid	0.5 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.025 mLD ₅₀ /mL
liquid egg	liquid	0.75 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.5 mLD ₅₀ /mL	0.05 mLD ₅₀ /mL
yogurt	semisolid	0.25 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL
broccoli	solid	0.25 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL
salami	solid	0.25 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL
salmon	solid	0.25 mLD ₅₀ /mL	0.1 mLD ₅₀ /mL	0.25 mLD ₅₀ /mL	0.01 mLD ₅₀ /mL

^aRequires pH adjustment.Table 3. Detection of BoNT/A, /B, /E, and /F Spiked at Levels Below 1 mLD₅₀ in 44 Additional Food Matrices

food	food state	BoNT/A	BoNT/B	BoNT/E	BoNT/F
beef stew	solid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
butter beans	solid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
carrot juice	liquid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
celery	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
cereal	solid	0.75 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
chicken soup	solid	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
chili	solid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
chili sauce ^a	liquid	0.75 mLD ₅₀	0.75 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
collard greens	solid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
cooked egg	solid	0.5 mLD ₅₀	0.25 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
corn cake	solid	0.75 mLD ₅₀	0.25 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
corn syrup	liquid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
cranberry juice ^a	liquid	0.5 mLD ₅₀	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
dried beef	solid	0.25 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
figures	solid	0.75 mLD ₅₀	0.75 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
garlic	solid	0.5 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
ham	solid	0.5 mLD ₅₀	0.75 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
honey	liquid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
jelly ^a	semisolid	0.75 mLD ₅₀	0.75 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
kim chi ^a	solid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
mackerel	solid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
molasses	liquid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
okra	solid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
onions	solid	0.5 mLD ₅₀	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
pebre	semisolid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
pickled peppers ^a	solid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
potato	solid	0.25 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
potato soup	semisolid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
refried beans	semisolid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
rice	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
salad dressing	liquid	0.75 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
salsa ^a	semisolid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
sauerkraut ^a	solid	0.5 mLD ₅₀	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
sofrito	semisolid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
sour cream dip	semisolid	0.5 mLD ₅₀	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
spaghetti	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
spaghetti sauce	semisolid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
squash	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
steak	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
tofu	semisolid	0.5 mLD ₅₀	0.5 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀
tomatoes ^a	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
tortillas	solid	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀	0.25 mLD ₅₀
vegemite	semisolid	0.75 mLD ₅₀	0.5 mLD ₅₀	0.5 mLD ₅₀	0.25 mLD ₅₀
whipping cream	semisolid	0.75 mLD ₅₀	0.25 mLD ₅₀	0.75 mLD ₅₀	0.25 mLD ₅₀

^aRequires pH adjustment.

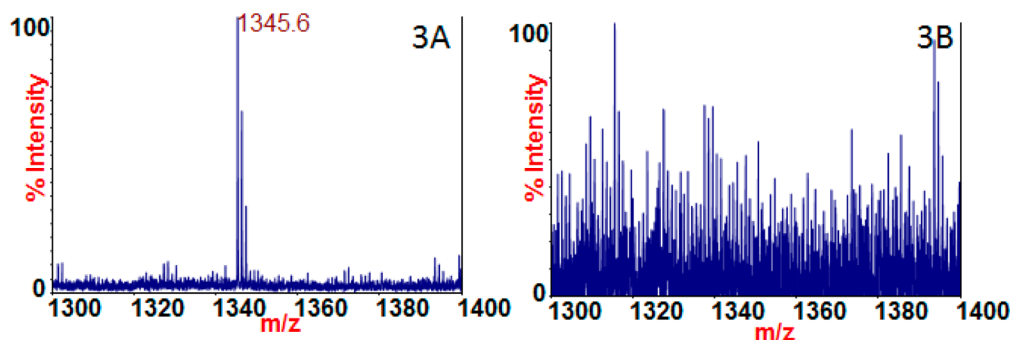


Figure 3. Mass spectra of Endopep-MS reactions containing (A) 0.01 mL_D₅₀ of BoNT/F spiked into cooked egg and (B) 0.01 mL_D₅₀ of BoNT/F in raw egg.

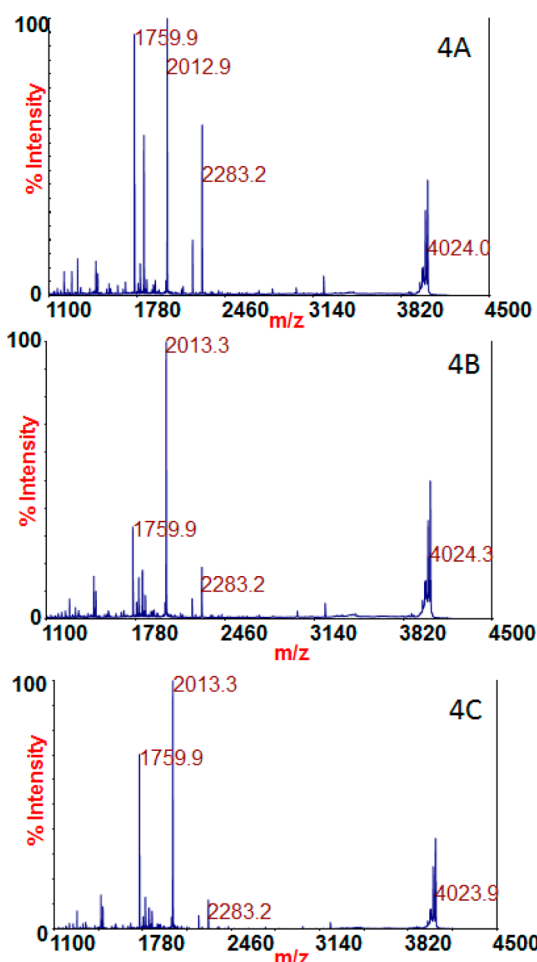


Figure 4. Mass spectra of Endopep-MS reactions containing BoNT/B spiked into (A) PBST, (B) orange juice with no neutralization, and (C) orange juice with neutralization. All were stored for 6 weeks at 4 °C prior to testing.

(Figure 1A) with whole milk spiked with 10 mouse LD₅₀ (mLD₅₀) of BoNT/A (Figure 1B) shows two new peaks in the spiked sample which are absent in the untreated sample. These peaks circled in red at *m/z* 999 and 1427 correspond to the cleavage of SubA by BoNT/A, proving the presence of enzymatically active BoNT/A in the milk. The uncleaved substrate (SubA) is present at *m/z* 2406. Through repeated analysis, we could reliably detect as little as 0.25 mL_D₅₀ of BoNT/A spiked into 1 mL of whole milk (Figure 2B).

BoNT/B, /E, and /F were also spiked into whole milk. By examining the differences between untreated whole milk (Figure 1C, 1E, and 1G) and whole milk spiked with 10 mL_D₅₀ of BoNT/B (Figure 1D), BoNT/E (Figure 1F), and BoNT/F (Figure 1H), the spiked milk can be distinguished from the untreated milk in all cases. In the case of BoNT/B, the two peaks at *m/z* 1760 and 2283 in Figure 1D correspond to cleavage of SubB by BoNT/B, peaks at *m/z* 1133 and 2501 in Figure 1F correspond to cleavage of SubE by BoNT/E, and peaks at *m/z* 1346 and 3784 in Figure 1H correspond to cleavage of SubF by BoNT/F. The uncleaved substrates are present at *m/z* 4025 for SubB, 3614 for SubE, and 5511 for SubF. Replicate analysis of whole milk spiked with BoNT/B, /E, and /F determined that we could detect as little as 0.1 mL_D₅₀ of BoNT/B (Figure 2D), 0.1 mL_D₅₀ of BoNT/E (Figure 2F), or 0.01 mL_D₅₀ of BoNT/F (Figure 2H) spiked into 1 mL of whole milk. Intact SubF and the N-terminal cleavage product produced by cleavage of the substrate by BoNT/F are long peptides with molecular weights of 5510 and 3783 Da and historically are rarely seen by MALDI-TOF/MS. However, the doubly charged intact SubF is often visible at *m/z* 2556, using the analytical instrumentation listed here.

Detection of BoNT/A, /B, /E, and /F in Additional Foods. BoNT/A, /B, /E, and /F were spiked into infant formula, yogurt, broccoli, green bean liquid, salami, salmon, liquid egg, orange juice, and tomato juice. Through repeated analysis, we determined that the limits of detection of BoNT/A, /B, /E, and /F spiked into 1 mL of these matrices is below the limit of detection of the mouse bioassay, 1 mL_D₅₀, as seen in Table 2. The limits of detection ranged from 0.01 mL_D₅₀ of BoNT/F in infant formula, yogurt, broccoli, green bean liquid, salami, and salmon up to 0.75 mL_D₅₀ of BoNT/A in liquid egg.

Additional foods which were of interest as they had been investigated in previous years as suspect agents of foodborne botulism were also tested with the Endopep-MS assay. For these foods, BoNT/A, /B, /E, and /F at levels below the limit of detection of the mouse bioassay were spiked into 0.5 mL of the liquid food or food extract. The results listed in Table 3 indicate that BoNT/A, /B, /E, and /F were successfully detected in 44 additional foods at levels below that of the historical standard, the mouse bioassay.

Many of the food matrices presented no challenges for the Endopep-MS assay. The results from many viscous matrices, such as corn syrup, honey, and molasses, looked identical to the results in PBST. On the basis of the similar results in buffer, it does not appear that the antibody-coated beads lacked sufficient mobility in the viscous matrix to capture the toxin nor does it seem that any of the toxin/antibody-coated beads

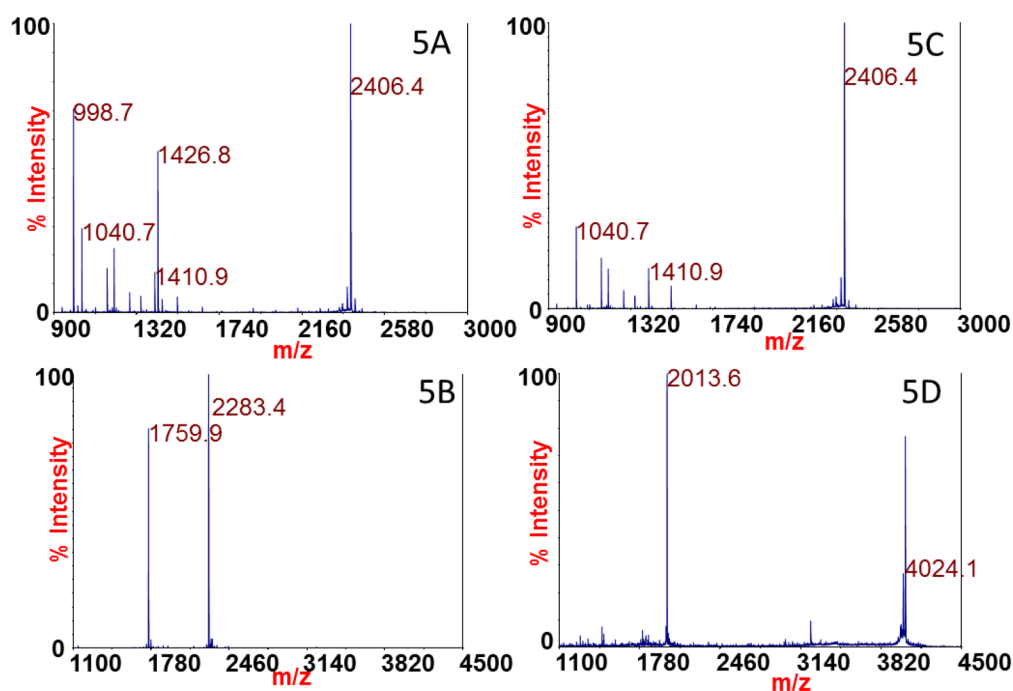


Figure 5. Mass spectra of Endopep-MS reactions testing for (A) BoNT/A and (B) BoNT/B in baked potato. Negative controls of baked potato were also tested for (C) BoNT/A and (D) BoNT/B.

remained in the viscous matrix. Samples with low pH such as tomato juice, salsa, chili sauce, kimchi, pickled peppers, and sauerkraut also looked similar to the results in PBST provided that the sample was neutralized with ammonium hydroxide to physiological pH prior to antibody–bead addition (data not shown).

Foods which were protease rich such as steak, dried beef, beef stew, and ham benefited greatly from washing the antibody-coated beads post toxin capture with 2 M NaCl to remove all nonspecific binding proteases from the antibody-coated beads. This technique was developed for the high protease environment of stool extracts,²⁶ so it is not surprising that it works for other protease-rich matrices. The high-salt washes however had a negative effect on high-fat matrices. Although it was possible to detect BoNT/A, /B, /E, and /F spiked into high-fat matrices such as mackerel, salami, salad dressing, and salmon when the antibody-coated beads were washed in 2 M NaCl post toxin capture, the limit of detection of the assay with these high-fat matrices was improved by washing the beads in PBST only (data not shown).

One of the more problematic matrices was the liquid egg matrix. Although the Endopep-MS assay could detect BoNT/A, /B, /E, and /F spiked into liquid egg at levels below the limit of detection of the mouse bioassay, the limit of detection in that matrix was elevated when compared to the other matrices for which a limit of detection was established (Table 2). It appeared that some of the antibody-coated beads remained behind in the matrix and the beads which were removed from the matrix had a clumpy appearance similar to the appearance of the beads from high-fat matrices when washed in 2 M NaCl. Altering the wash buffers from 2 M NaCl to PBST did not improve the appearance of the beads when used with the liquid egg matrix.

It was suspected that perhaps the high level of biotin in the liquid egg interfered with the antibody-capture process as the beads used for antibody-capture are streptavidin coated and

biotin has a very strong affinity for streptavidin. Upon switching to protein-G-coated beads which do not contain streptavidin, however, similar limits of detection were obtained, indicating that the high level of biotin in eggs did not cause the bead retention or clotted appearance. Additionally, when BoNT was spiked into the extract of cooked eggs, lower levels of toxin could be detected. As little as 0.01 mLD₅₀ of BoNT/F spiked into 1 mL of cooked egg extract produced a visible C-terminal cleavage product indicating the presence of active BoNT/F (Figure 3A); however, cleavage product was absent when the same level of toxin was spiked into 1 mL of liquid raw egg (Figure 3B). A level of 0.01 mLD₅₀ of BoNT/F could be detected by diluting the liquid egg matrix 1:10 in PBST; however, this step would negatively impact the limit of detection by an order of magnitude in a real outbreak sample as the limit of detection would be increased to 0.1 mLD₅₀/mL of undiluted sample. Because as little as 0.05 mLD₅₀ of BoNT/F could be detected in 1 mL of undiluted liquid egg (Table 2), a lower limit of detection was achieved by using 1 mL of liquid egg matrix with no dilution.

Another of the matrices with an increased limit of detection was orange juice (Table 2). Orange juice is very acidic, and it was thought that the low pH of the matrix had an effect on either the enzymatic activity of the toxin or the capture of the toxin by the antibody. An amount of one mLD₅₀ of BoNT/B was spiked into 1 mL of orange juice or PBST and stored for 6 weeks at 4 °C. Endopep-MS of the samples proceeded both with and without neutralization of the pH of the orange juice immediately prior to antibody–bead addition. The peaks at *m/z* 1760 and 2283 in Figure 4 correspond to BoNT/B cleavage of SubB, illustrating that even after being stored for 6 weeks at 4 °C, active toxin remained in all three samples. However, comparison of the intensity of the cleavage product peaks (particularly the peak at *m/z* 1760) compared to the intensity of the uncleaved SubB peak (at *m/z* 4024 and especially 2013) indicates that there is either less toxin or less active toxin in the

untreated orange juice sample (Figure 4B) and, to a lesser degree, the neutralized orange juice sample (Figure 4C) when compared to the PBST sample.

Application of Endopep-MS to Foods Suspected of Causing Botulism Outbreaks. Thirty-one food extracts received for testing during botulism outbreak investigations were tested by Endopep-MS for BoNT/A, /B, /E, and /F in parallel with the mouse bioassay. Twenty-nine of these samples were below the limit of detection of BoNT/A, /B, /E, and /F with Endopep-MS as well as the mouse bioassay. One chili pepper sample tested positive for BoNT/B by Endopep-MS, and based on the intensity of the BoNT/B cleavage products, the level of toxin was very low, near the limit of detection for the Endopep-MS assay. This sample tested below the limit of detection of the mouse bioassay. The baked potato sample tested positive for both BoNT/A and /B by Endopep-MS as seen in Figure 5.

Peaks at m/z 999 and 1427 in Figure 5A indicate the presence of active BoNT/A in this sample, and peaks at m/z 1760 and 2283 indicate the presence of active BoNT/B in this same sample (Figure 5B), compared to the negative control of baked potato tested for BoNT/A (Figure 5C) and BoNT/B (Figure 5D). Mouse bioassay results on these food samples were identical to the Endopep-MS results. The finding of two serotypes of BoNT in a single sample is rare, although not completely unique as some strains of *C. botulinum* are known to make more than one BoNT serotype. These are called bivalent toxin producers and are named Ab, Ba, Af, or Bf, with the capital letter representing what is thought to be the main serotype produced. Upon the basis of the intensity of the BoNT/A and /B cleavage products, it would appear that the baked potato contained a Ba strain of *C. botulinum*. Alternatively, the potato could have been contaminated with 2 strains of *C. botulinum*.

In conclusion, we demonstrated that the Endopep-MS method is able to detect enzymatically active BoNT/A, /B, /E, and /F in a wide variety of foods. These foods represent different characteristics such as low pH, high viscosity, high fat, and varied ethnicities. Many of the foods were suspected of causing a botulism outbreak in the past, and others have definitively caused prior botulism outbreaks. The limit of detection varies and is matrix and serotype dependent, but all matrices tested were able to detect BoNT/A, /B, /E, and /F spiked into foods at levels below the limit of the historical standard assay for botulism, the mouse bioassay. The method accurately identified BoNT/A and BoNT/B in a baked potato associated with a recent case of botulism. The combination of the ability to detect BoNT/A, /B, /E, and /F in foods as well as clinical specimens such as stool extracts make this assay a concrete alternative to the mouse bioassay for BoNT detection.

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Notes

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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