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(54) Title: PEPTIDES, ANTIBODIES, AND METHODS FOR DETECTION OF BOTULINUM NEURITOXIN A SUBTYPES

(57) Abstract: The invention provides a mass spectrometry method able to identify BoNT/A subtypes in complex matrices, including crude culture supernatants and food, environmental, and biological samples. Peptides from the BoNT light chain (Lc) specific for BoNT/A subtypes were identified. The method comprises an immunocapture step with antibodies specific for BoNT/A Lc chains followed by mass spectrometry, preferably liquid chromatography tandem mass spectrometry (LC-MS/MS) on a triple quadrupole mass spectrometer (QqQ) in multiple reaction monitoring (MRM) mode. In addition to the detection of peptides from the BoNT light chain (Lc) specific for BoNT/A subtypes, peptides from the BoNT heavy chain (Hc) specific for BoNT/A subtypes can also be detected.



**PEPTIDES, ANTIBODIES, AND METHODS FOR DETECTION OF
BOTULINUM NEUROTOXIN A SUBTYPES**

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention relates to the fields of medicine, public health, and public safety. More specifically, the present invention relates to peptides, antibodies, and diagnostic methods for detecting *Clostridium botulinum* neurotoxin A (BoNT/A) subtypes.

10 Description of Related Art

Botulinum neurotoxins (BoNTs) are the most potent toxins and are responsible for a severe paralytic disease, botulism, in man and animals. Most often, botulism occurs subsequently to the ingestion of food contaminated with *Clostridium botulinum* and containing preformed BoNT. The toxin is absorbed from the intestine, and reaches the neuromuscular
15 junction via the blood or lymph circulation and the interstitial fluid. BoNTs act at the neuromuscular junction by inhibiting the evoked release of acetylcholine, thus leading to paralytic symptoms, including blurred vision, ptosis, dry mouth, difficulty in swallowing, then descending flaccid paralysis of voluntary muscles and respiratory insufficiency (1-3).

BoNTs are divided into seven toxinotypes (A to G) based on their immunological
20 properties using the mouse biological test and specific neutralizing antisera (4,5). BoNT genes (*bont*) have been sequenced from a large number of strains and sequence comparison has permitted researchers to identify sequence variations in each toxinotype. Additionally, botulinum toxinotypes are divided into subtypes (6-8). Five BoNT/A subtypes (A1 to A5) have been identified and recently three additional subtypes (A6 to A8) have been reported (9-11)
25 (GenBank Accession Numbers FJ981696, JQ954969, KF667385, respectively). The significance of sequence variation in each toxinotype is not yet fully understood, but the variation impacts diagnostic tests and development of therapeutic agents, such as those based on immunotherapy, as previously discussed (12). Antigenic differences have been found between BoNT/As produced by *C. botulinum* A strains isolated from infant botulism and food-borne
30 botulism (13). BoNT/A1 and BoNT/A2, which differ by 10% at the amino acid sequence level, show substantial differences in monoclonal antibody-binding affinity. Among six monoclonal antibodies that bind to BoNT/A1 with high affinity, three show a marked decrease in binding affinity (500- to more than 1000-fold decrease) to BoNT/A2 (14). Therefore, *in vivo*

neutralization of a BoNT toxinotype that includes the intra-toxinotype variants requires combinations of monoclonal antibodies that tightly bind and potently neutralize each subtype. As such, an association of three monoclonal antibodies with high affinity binding to subtypes A1 and A2 can completely neutralize A1 or A2 toxin. However, replacement of two of the three
5 monoclonal antibodies with two having a low binding affinity to BoNT/A2 induces a decrease in BoNT/A2 neutralization (50-fold less binding) (15). Sequence variation among subtypes might also affect the toxin activity and subsequently the course of the disease.

The enzymatic site of BoNT/A3 and BoNT/A4 light chain (Lc) is conserved, but non-conservative mutations in domains involved in substrate (SNAP-25) recognition result in
10 different activity (14). Although Lc chains from subtypes A1 to A4 bind to the substrate SNAP25 with similar affinity, the Lc chain from subtype A4 and to a lower extent from subtype A3, cleaves SNAP25 less efficiently than Lc chain subtypes A1 and A2 (2 and 23 fold less, respectively) (16,17). BoNT/A1 to A5 exhibit distinct *in vitro* catalytic activity as well as different biological activity in neuronal cell and mouse models (18). BoNT/A2 enters neuronal
15 cells faster than BoNT/A1 and it is more potent (19). In addition, BoNT/A2 is a more potent inhibitor of neurotransmission at the neuromuscular junction (20). However, both toxins have comparable duration of action (21).

BoNT sequence variation also is involved in protection by vaccination. As tested in mice, subtypes A1, A2, and A3, whole BoNT, or a subunit vaccine based on the C-terminal half
20 of the heavy chain induce efficient homologous protection, but variable cross-protection according to the subtype (22).

Development of vaccines and neutralizing antibodies against all the subtypes of *C. botulinum* is a major issue in prevention and treatment of botulism. The recent controversy about the reported new BoNT type H, also referred as a new hybrid F/A, is mainly based on the
25 existence or absence of cross-protection with the already known BoNT types and subtypes, and further shows the importance of subtype determination (23-25).

Subtype identification is usually performed via PCR amplification of *bont* genes and subsequent DNA sequencing (6,26). This method requires the presence of the toxigenic strain in the samples and optimally strain isolation and DNA extraction. However, certain samples might
30 contain BoNT in the absence of the bacterium, making it impossible to use traditional DNA-based methods.

Mass spectrometry (MS) methods have been developed to detect active BoNTs through peptide identification from substrate cleavage (Endopep-MS) (reviewed in (27)). Endopep-MS is a sensitive method to detect BoNT types, but it cannot identify the toxin subtypes because all of the subtypes of each type recognize the same cleavage site.

5 Tryptic digestion of the toxin itself and analysis of tryptic peptides by MS might allow subtype determination. Using that technique, BoNT/A1 and BoNT/A2 have been identified in spiked milk samples based on differential peptides between the two subtypes (28). In that method, holoneurotoxin in the spiked sample was bound by an antibody specific for the heavy chain of the holoneurotoxin. The entire holoneurotoxin was digested with trypsin, and all trypsin
10 fragments were subjected to matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) or liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis, followed by searching a protein sequence database to identify the subtype in the sample. According to that method, 58% of the theoretical differences between BoNT/A1 and BoNT/A2 were identified. A similar approach has been described to differentiate the subtypes of BoNT/B (29).

15 A multiple enzyme and sequential in-gel digestion approach (MESID) also has been proposed for BoNT subtyping. The multiple proteolytic enzymes used in this method facilitate near-complete sequence coverage of all the seven BoNT toxinotypes (30).

Prior MS techniques using BoNT heavy chains for analysis are complicated by the large size and large number of trypsin cleavage products. Although various methods have been used
20 to detect and differentiate *C. botulinum* neurotoxin subtypes, there continues to be a need in the art for improved techniques that are faster and more accurate.

SUMMARY OF THE INVENTION

Here, the inventors designed a mass spectrometry method able to identify BoNT/A
25 subtypes in complex matrices, including crude culture supernatants and food, environmental, and human serum samples, or in pharmaceutical or cosmetic products. The complex matrices can include multiple subtypes in the same sample. Using the method, peptides from the BoNT light chain (Lc) specific for the subtypes BoNT/A1 to A3 and BoNT/A5 to A8 were identified. The method comprises an immunocapture step with antibodies specific for BoNT/A Lc chains
30 followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a triple quadrupole mass spectrometer (QqQ) in multiple reaction monitoring (MRM) mode. BoNT/A subtypes were correctly identified in culture supernatants and in tap water or orange juice

samples with a limit of detection of 20 to 150 mouse lethal doses (MLD) and with a lower sensitivity in serum samples. Because of the conserved nature of BoNT/A Lc chains, it was unexpected that they could serve as a basis for the discrimination of all of the BoNT/A subtypes.

In a first aspect, the invention provides a method for determining one or more
5 *Clostridium botulinum* neurotoxin type A (BoNT/A) subtypes in a sample. In general, the method comprises contacting the light chain of the BoNT/A subtype neurotoxin with an antibody that is specific for a conserved sequence among all BoNT/A subtype light chains, digesting the light chain with trypsin to generate multiple trypsin fragments, and subjecting the trypsin fragments to mass spectrometry, preferably liquid chromatography-triple quadrupole mass
10 spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS), to determine the sequences of the fragments. In one embodiment, the method comprises detecting a peptide consisting of the amino acid sequence of any one of SEQ ID NOs 2 and 10-42. Because the trypsin digestion is performed on only the light chain, and because the light chain is substantially smaller than the heavy chain, the complex analysis of the sequences of the tryptic fragments is vastly simplified
15 compared to prior art methods using the heavy chain. Furthermore, because there are relatively few tryptic fragments as compared to prior art methods, the present method is capable of detecting multiple subtypes in the same sample.

In another aspect, the invention provides an antibody that specifically binds to a conserved region of the light chain among all subtypes of BoNT/A. Preferably, the antibody
20 specifically binds to peptide Q67-K84 consisting of the amino acid sequence QVPVSYDSTYLSTDNEK (SEQ ID NO:3) or peptide D49-K66 consisting of the amino acid sequence DTFTNPEEGDLNPPPEAK (SEQ ID NO:4). In preferred embodiments, the antibody is bound to a solid support to facilitate separation of the light chain from other substances in a sample in which it is found.

25 In yet another aspect, the invention is directed to kits for determining a BoNT/A subtype in a sample. The kit includes an antibody according to the invention, and can include additional reagents and supplies for practicing the method according to the invention.

In yet another aspect, the invention provides a method for determining one or more
30 *Clostridium botulinum* neurotoxin type A (BoNT/A) subtypes in a sample by contacting the heavy chain of the BoNT/A subtype neurotoxin with an antibody that is specific for a conserved sequence among all BoNT/A subtype heavy chains and/or contacting the light chain of the BoNT/A subtype neurotoxin with an antibody that is specific for a conserved sequence among all

BoNT/A subtype light chains, digesting the heavy and light chains with trypsin to generate multiple trypsin fragments, and subjecting the trypsin fragments to mass spectrometry, preferably liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS), to determine the sequences of the fragments. In one embodiment, the method comprises detecting a peptide consisting of the amino acid sequence of any one of SEQ ID NOs 2, 10-42 and 44. The present method is capable of detecting multiple subtypes in the same sample.

The invention also provides an antibody that specifically binds to a conserved region of the heavy chain among all subtypes of BoNT/A. Preferably, the antibody specifically binds to the peptide consisting of the amino acid sequence

GPLGSSTDIPFQLSKYVDNQRLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK
VNFDPIDKNQIQLFNLESSKIEVILKNAIVNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
CMENNSGWKVSLSNYGEIHWTLQDTQEIKQRVVFVKYSQMINISDYINRWIFVTITNNRLNN
SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL
YDNQSN SGILKDFWGDYLYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV
MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL
VASNWYNRQIERSSRTLGCSEWIFVDDGWGERPL (SEQ ID NO:47).

In yet another aspect, the method of the invention further comprises a step for quantification of the BoNT/A subtype by using mass spectrometry, preferably liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS), with an internal and isotopically stable standard.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention, and together with the written description, serve to explain certain principles of the invention.

Fig. 1. shows an example of detection of a conserved peptide in culture supernatants from representative *C. botulinum* A subtypes using ion chromatography. The figure shows ion chromatograms of SRM transition 565.8/684.4 (LYGIAINPNR (SEQ ID NO:1) peptide) from BoNT/A1 (strain Hall sample, equivalent to 10^4 MLD100), BoNT/A2 (strain 9336, 10^5 MLD100), BoNT/A3 (strain Loch Maree, 10^3 MLD100), BoNT/A5 (strain 126.07, 10^4

MLD100), BoNT/A7 (strain 148.08, 10^4 MLD100), and BoNT/A8 (strain 217.12, 8×10^3 MLD100) supernatants. *Clostridium sporogenes* (strain 261.05) supernatant was used as the negative control. The expected retention time is 15.7 min.

Fig. 2 shows an example of detection BoNT/A2 in culture supernatants from representative *C. botulinum* A subtypes using ion chromatography. The figure shows ion chromatograms of SRM transition 424.2/215.1 (DVASTLNK (SEQ ID NO:2) peptide) from BoNT/A1 (strain Hall sample, equivalent to 10^4 MLD100), BoNT/A2 (strain 9336, 10^5 MLD100), BoNT/A3 (strain Loch Maree, 10^3 MLD100), BoNT/A5 (strain 126.07, 10^4 MLD100), BoNT/A7 (strain 148.08, 10^4 MLD100), and BoNT/A8 (strain 217.12, 8×10^3 MLD100) supernatants. *Clostridium sporogenes* (strain 261.05) supernatant was used as the negative control. The expected retention time is 10.1 min.

Fig. 3 shows an example of detection of BoNT/A2 at the Limit Of Detection (LOD) level using ion chromatography. The figure shows ion chromatograms of SRM transition 421.2/215.1 (DVASTLNK (SEQ ID NO:2) peptide) from BoNT/A2 (strain 9336, 30 MLD100) and matrix blank (TGY medium).

Fig. 4 shows a schematic representation of the main nomenclatures used in mass spectrometry.

Fig. 5 shows an example of the determination of the linearity limit in relation to the quantity of BoNT/A1 light chain present in a sample (200 to 6400 ng).

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS OF THE INVENTION

Reference will now be made in detail to various exemplary embodiments of the invention. Data supporting the embodiments are presented in the accompanying figures. It is to be understood that the following discussion of exemplary embodiments is not intended as a limitation on the invention, as broadly disclosed herein. Rather, the following discussion is provided to give the reader a more detailed understanding of certain aspects and features of the invention.

Before embodiments of the present invention are described in detail, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Further, where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit,

unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither, or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the term belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The present disclosure is controlling to the extent it conflicts with any incorporated publication.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a BoNT/A subtype" includes a plurality of such subtypes or to "the antibody" includes reference to one or more antibodies, and so forth. Furthermore, the use of terms that can be described using equivalent terms include the use of those equivalent terms. Thus, for example, the use of the term "tube" is to be understood to include the terms "vial", "bottle", and other terms used in the art to indicate a container for holding or manipulating a sample under investigation.

One aspect of the invention is directed to a method for determining a *C. botulinum* neurotoxin type A (BoNT/A) subtype in a sample. Such method is accordingly an *in vitro* method for determining a *C. botulinum* neurotoxin type A (BoNT/A) subtype in a sample. Broadly speaking, the method comprises contacting the light chain of the BoNT/A subtype neurotoxin with an antibody that is specific for an amino acid sequence that is conserved among BoNT/A subtypes, enzymatically cleaving the light chain, preferably with trypsin, to generate multiple fragments of the light chain (*e.g.*, trypsin fragments), and subjecting the fragments (*e.g.*, trypsin fragments) to mass spectrometry, preferably liquid chromatography-triple quadrupole

mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS), to determine the sequences of the fragments (e.g., trypsin fragments). Comparison of the sequences of the fragments, and in particular the sequences of the fragments containing regions that are variable among the BoNT/A subtypes, to known sequences of BoNT/A subtypes allows identification of the BoNT/A subtype in the sample. In embodiments, the method can be considered a method of detecting a BoNT/A subtype in a sample, i.e. is an *in vitro* method of detecting a BoNT/A subtype in a sample.

The method of the invention can be practiced on any sample. Typically, the sample is one containing biological material and is suspected of containing a BoNT/A subtype neurotoxin.

Non-limiting examples of samples include food, such as food intended for human consumption. For example, the food can be honey, home-canned vegetables and fruits, corn syrup, improperly canned commercial foods, fish, herb-infused oils, baked potatoes, cheese sauce, bottled garlic, and any food that is allowed to stay warm for extended periods of time. Other non-limiting examples of samples include liquids, such as water or biological samples, such as serum, stool, and gastric juice. Additionally, the sample can be an environmental sample, such as a soil sample or a sample of water from a lake, stream, river, etc. or pharmaceutical or cosmetic product. Preferably, the sample is a pharmaceutical or cosmetic product, in particular is obtained from a manufacturing lot or batch release. One advantage of the present invention is that the sample does not need to have viable neurotoxin-producing bacteria or DNA from the *C. botulinum* subtype to be determined. The method allows for detection of BoNT/A subtype sequences in samples in ranges of concentrations typically seen in food and environmental samples.

According to another aspect, the method of the invention can be used to detect BoNT/A subtype sequences present as active ingredient in a pharmaceutical or cosmetic product, in particular a manufacturing lot or batch release, including to check the quality (e.g. degree of purity) or the quantity of the produced BoNT/A, in particular BoNT/A1, for use in pharmaceutical or cosmetic applications (*Botulinum Toxin as a Clinical Product: Manufacture and Pharmacology*", Andy Pickett, Chapter 2, *Clinical Applications of Botulinum Neurotoxin, Current Topics in Neurotoxicity 5*, K. A. Foster (ed.), DOI 10.1007/978-1-4939-0261-382).

Accordingly the method of the invention can also be carried out during the process of preparation of the active ingredient consisting of BoNT/A.

The antibody used in the method can be any polyclonal or monoclonal antibody that specifically binds to an epitope on the light chain of a BoNT/A neurotoxin that is conserved among all BoNT/A subtypes. In a non-limiting example described below, the antibody is a polyclonal antibody raised against the amino acid sequences QVPVSYDSTYLSTDNEK (SEQ ID NO:3) and DTFTNPEEGDLNPPPEAK (SEQ ID NO:4). Preferably, at some point, the antibody is bound to a solid support, such as a magnetic bead. Methods for raising antibodies and binding antibodies to solid supports are well known in the art and thus need not be detailed here.

The antibody is capable of specifically binding to the light chain of all BoNT/A subtypes present in the sample. Binding to the light chain can occur in the context of the holoneurotoxin or after dissociation of the light chain from the heavy chain of the neurotoxin. After binding of the light chain to the antibody and dissociation of the light chain from the heavy chain, and separation of the light chain from the heavy chain, the light chain is denatured and preferably separated from the antibody/solid support. Then, the light chain is digested (*e.g.*, using trypsin) to create fragments (*e.g.*, trypsin fragments), and the fragments (*e.g.*, trypsin fragments) are analyzed using mass spectrometry, preferably LC-QqQ-MS/MS.

Comparison of the sequences of the fragments allows for identification of the presence and identity of one or more particular BoNT/A subtypes in the sample. Upon confirmation using positive and negative controls, lack of detection of BoNT/A subtype light chain sequences indicates the absence of BoNT/A subtype light chains in the sample, or presence in an amount below the detection limits of the method.

The method of the present invention is based, at least in part, on certain unexpected findings. Prior to developing the present method, it was uncertain whether targeting the light chain for antibody binding and MS detection of discriminatory peptides would be successful. Prior attempts to identify or differentiate BoNT/A or BoNT/B subtypes relied upon antibody capture via binding to the heavy chain of BoNT/A or BoNT/B neurotoxins. The light chain is more conserved than the heavy chain. Therefore, it was not possible to predict that targeting the light chain would be successful in discriminating all of the BoNT/A subtypes. In addition, prior to developing the present method, one could not predict with any level of confidence that it would be possible to identify multiple different subtypes in a single sample.

To practice the method of the present invention, an antibody that specifically binds to an amino acid sequence of the BoNT/A light chain that is conserved among all BoNT/A subtypes is

used. As mentioned above, the antibody can be a polyclonal antibody or a monoclonal antibody. The precise sequence of the conserved region is not critical to practice of the invention. Rather, any conserved sequence present in all of the subtypes can be used to generate the antibody. For ease of use in the method of the invention, the antibody can be bound to a solid support either before or after binding of the antibody to the light chain. Preferably, the antibody is bound to the solid support before binding of the antibody to the light chain. Numerous solid supports are known to the skilled artisan, and any suitable solid support may be used. Non-limiting examples of solid supports include beads, such as magnetic beads, and strips or sheets, such as those made from nitrocellulose or nylon. Solid supports encompassed by the present invention are commercially available, and the skilled artisan is well aware of vendors of such solid supports and how to use them without the need for a detailed description herein.

In one embodiment, the invention comprises digesting the light chain with a proteolytic enzyme to create proteolytic fragments for mass spectrometry. Preferably, trypsin is used to generate trypsin fragments. Other non-limiting preferred enzymes include chymotrypsin and pepsin. The fragments can be analyzed using mass spectrometry, liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS). Other non-limiting mass spectrographic methods include LC-QqQ-MS/MS in MRM mode, LC-ESI-MS/MS, MALDI/QTOF, LTQ-FT, and LC-QTOF-MS. The method can comprise detecting a peptide consisting of the amino acid sequence of any one of SEQ ID NO 2 and 10-42, in particular a peptide consisting of the amino acid sequence of any one of SEQ ID NO 1, 2, 7, 10, 11, 15, 22, 28, 29, 31, 36, 37 and 40.

In another aspect, the invention provides a kit that is designed for practice of the method of the invention. In its basic form, the kit comprises a container that contains an antibody according to the invention. The antibody can be provided in a single container, which can include sufficient antibody to perform the method of the invention one or multiple times. Alternatively, the kit can contain multiple containers of antibody, each of which can include sufficient antibody to perform the method of the invention one or multiple times.

In embodiments, the kit includes additional reagents and supplies for practicing the method according to the invention. For example, in embodiments, the kit comprises a solid support to which the antibody is bound or to which the antibody can be bound. As with the antibody of the kit, the solid support (*e.g.*, magnetic beads) can be provided in one or multiple containers. Buffers, reagents, etc. for binding of antibody to the solid support and for separating

the solid support/antibody/light chain complexes from other substances present in the sample and from the heavy chain of the holoneurotoxin can also be included in the kit. Likewise, a negative control (*i.e.*, a sample known not to contain any BoNT/A subtype light chain material) can also be provided as part of the kit.

5 Regardless of the contents of the kit, the kit in general includes an overall container that allows for packaging of kit materials together. The overall container can be any of the various containers commonly used in biological assay kits, such as a box suitable for shipping and storing the kit contents. The overall container can be made of any suitable material, such as cardboard, plastic, or metal. The container can also include material that can be used to
10 safeguard the integrity of containers held within it. For example, the kit can include a foam insert to securely hold kit contents to minimize the chance of breakage of kit contents during shipping or storage.

 Detection of BoNT in biological samples and food is critical for early diagnosis of botulism and to apply an efficient treatment of the disease. Investigation of BoNT in food or
15 environmental samples is also essential for identifying the source of contamination (*e.g.*, poor food handling techniques, bioterrorism) and preventing outbreak extension of botulism. Typing and subtyping are important to estimate the severity of the disease and to adapt the appropriate treatment, notably the specificity of the antisera. The determination of typing and subtyping is also critical in botulism outbreak investigations and to trace the source of intoxication, allowing
20 determination of the commonality or difference between strains from patients of the same outbreak or distinct outbreaks, and between strains from patients and suspected food. Mass spectroscopy is a rapid and powerful method to identify proteins. Notably, this method allows one to get results in a few hours from a culture supernatant (about 6 hours) instead of a few days with molecular biology techniques, which require DNA preparation and sequencing, usually in a
25 external platform or company. The present invention provides data showing that an LC-QqQ-MS/MS method in MRM mode coupled to an immunopurification step allows identification of BoNT/A type and subtypes from complex samples. The method, based on Lc peptides specific for BoNT/A subtypes A1 to A8, yielded unambiguous identification of the subtypes. A previous MS approach has been designed to differentiate BoNT/A1 and BoNT/A2 (28). Identification of
30 BoNT types A1 to G was achieved by using multiple enzyme digestion in gel and subsequent LC-QqQ-MS analysis. That method is predicted to identify the subtypes but has not yet been validated (30). The present procedure efficiently discriminated the distinct BoNT/A subtypes.

One important advantage of the present method is that complex samples can be used. Subtyping was efficiently performed with crude culture supernatants. An additional advantage is that this method of subtyping is performed in one step without the need for further techniques or reagents to address the subtyping. The LOD of the present method, as exemplified below, was 20 to 150 equivalents MLD in 0.5 ml. This range is in the range of the minimal toxin concentrations found in food. The sensitivity of the method exemplified below of course could be increased by using a greater sample volume. Using artificially spiked samples representative of food, environmental, and biological samples, the LC-QqQ-MS/MS method of the present invention allowed the identification of all the BoNT/A subtypes tested. LOD varied with the matrix, with the highest values in serum samples. The exemplary method described below can be used with contaminated environmental or food samples, which usually contain significant BoNT concentrations (100 MLD/g and more). An advantage of the LC-QqQ-MS/MS approach is to allow subtype identification in the absence of viable neurotoxin-producing bacteria or available DNA in samples to perform a genetic subtyping. It is noteworthy that this methodology can be extended to identify new subtypes that will be evidenced by selecting additional specific peptides on light chains.

The method of the invention further comprises a step for quantification of the BoNT/A subtype by using MS/MS with an internal and isotopically stable standard (V.Brun ad al, Molecular and cellular Proteomics, 2007; 6(12) 2139-2149; Protein Standard for Absolute Quantification, PSAQTM; International patent application WO2008/145763).

In one embodiment, the invention provides a method for the quantification of a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:

- adding an internal standard to a sample comprising or suspected of comprising BoNT/A;
- reducing proteins in said sample;
- contacting said reduced sample with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, to isolate BoNT/A light chains;
- digesting the isolated light chains with a proteolytic enzyme to create proteolytic fragments of the light chains;
- subjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments; and
- quantitating the amount of BoNT/A subtype present in the sample.

In the method of quantification of the invention and to avoid false quantification, the internal standard is added to the sample before the treatment steps so that the BoNT/A subtype to be quantified and the internal standard follow the same treatment steps (immunocapture, digestion) and analysis steps.

5 In a particular embodiment, the invention provides a method for the quantification of a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:

adding an internal standard to a sample comprising or suspected of comprising BoNT/A;
reducing proteins in said sample;

10 contacting said reduced sample with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, wherein the antibody is coupled to a solid support, to form a solid support-antibody-light chain complex;

separating the solid support-antibody-light chain complex from other substances present in the sample;

15 digesting the light chains with trypsin to create trypsin fragments of the light chains;

separating solid support and antibody from the trypsin fragments;

subjecting the trypsin fragments to mass spectrometry, in particular liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS) to determine the sequences of the fragments; and

20 quantitating the amount of BoNT/A subtype present in the sample.

In another aspect, in addition to the detection of peptides from the BoNT light chain (Lc) specific for BoNT/A subtypes, peptides from the BoNT heavy chain (Hc) specific for BoNT/A subtypes can also be detected in a sample as previously defined. In this case, there is no need to separate the heavy and light chains of BoNT/A, and accordingly the sample is not subjected to a
25 denaturation step by heating and addition of a reducing agent such as betamercaptoethanol. Protocols previously described for the detection and optionally quantification of the BoNT/A light chains also apply for the BoNT/A heavy chains and/or light chains.

The inventors identified the peptide L823-R831 (LVASNWYNR (SEQ ID NO:43)) and the peptide T839-L858 (TLGCSWEFIPVDDGWGERPL (SEQ ID NO: 44)) from the heavy
30 chain (Hc) of BONT/A. The peptide L823-R831 is common to the subtypes A1 to A8 and the peptide T839-L858 is specific of the subtype A1.

In one embodiment, the method of the invention comprises contacting the heavy chain of the BoNT/A subtype with an antibody that is specific for an amino acid sequence that is conserved among BoNT/A subtypes and/or contacting the light chain of the BoNT/A subtype with an antibody that is specific for an amino acid sequence that is conserved among BoNT/A subtypes, enzymatically cleaving the recovered and optionally isolated BoNT/A or the heavy and light chains, preferably with trypsin, to generate multiple digestion fragments of the heavy and light chains, and subjecting the fragments to mass spectrometry to determine the sequences of the fragments. Comparison of the sequences of the fragments, and in particular the sequences of the fragments containing regions that are variable among the BoNT/A subtypes, to known sequences specific for BoNT/A subtypes allows identification of the BoNT/A subtype in the sample. In embodiments, the method can be considered a method of detecting a BoNT/A subtype in a sample.

In one embodiment, the invention comprises digesting the heavy and light chains with a proteolytic enzyme to create proteolytic fragments for mass spectrometry. Preferably, trypsin is used to generate trypsin fragments. Other non-limiting preferred enzymes include chymotrypsin and pepsin. The fragments can be analyzed using mass spectrometry, liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS). Other non-limiting mass spectrographic methods include LC-QqQ-MS/MS in MRM mode, LC-ESI-MS/MS, MALDI/QTOF, LTQ-FT, and LC-QTOF-MS. The method can comprise detecting a peptide consisting of the amino acid sequence of any one of SEQ ID NO 2, 10-42 and 44, in particular a peptide consisting of the amino acid sequence of any one of SEQ ID NO 1, 2, 7, 10, 11, 15, 22, 28, 29, 31, 36, 37, 40 and 44.

Antibodies used in the above-mentioned method to recover BoNT/A or its heavy chain and its light chain sequences can be any polyclonal or monoclonal antibodies that specifically bind to an epitope on the heavy chain of a BoNT/A neurotoxin that is conserved among all BoNT/A subtypes and/or an epitope on the light chain of a BoNT/A neurotoxin that is conserved among all BoNT/A subtypes. In a non-limiting example described below, the antibody is a polyclonal antibody raised against the amino acid sequence

GPLGSSTDIPFQLSKYVDNQRLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK
VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
CMENNSGWKVS LNYGEIHWTLQDTQEIKQRVVFYKYSQMINISDYINRWIFVTITNRLNN
SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL

YDNQNSNGILKDFWGDYLYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPGRGSV
 MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
 EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNIAKL
 VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47). Preferably, at some

5 point, the antibody is bound to a solid support, such as a magnetic bead. Methods for raising antibodies and binding antibodies to solid supports are well known in the art and thus need not be detailed here.

The invention relates in particular to the following embodiments:

10 Embodiment 1. A method for determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:

contacting a sample comprising or suspected of comprising BoNT/A with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, to isolate BoNT/A light chains;

15 digesting the isolated light chains with a proteolytic enzyme to create proteolytic fragments of the light chains; and

subjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments.

Embodiment 2. The method of embodiment 1, comprising detecting by mass
 20 spectrometry a peptide consisting of the amino acid sequence of any one of SEQ ID NOs 2 and 10-42.

Embodiment 3. The method of embodiment 1 or 2, wherein the method comprises:
 reducing proteins in the sample;

25 contacting the sample comprising or suspected of comprising BoNT/A with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, wherein the antibody is coupled to a solid support, to form a solid support-antibody-light chain complex;

separating the solid support-antibody-light chain complex from other substances present in the sample;

digesting the light chains with trypsin to create trypsin fragments of the light chains;

30 separating solid support and antibody from the trypsin fragments; and

subjecting the trypsin fragments to mass spectrometry, in particular liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS) to determine the sequences of the fragments.

Embodiment 4. A method for determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:

contacting a sample comprising or suspected of comprising BoNT/A with antibodies specific for conserved heavy chain sequences and/or antibodies specific for conserved light chain sequences among BoNT/A subtypes, to isolate BoNT/A or the BoNT/A heavy and light chains;

digesting the isolated BoNT/A or the isolated heavy and light chains with a proteolytic enzyme to create proteolytic fragments of the heavy and light chains; and

subjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments.

Embodiment 5. The method of embodiment 4, comprising detecting by mass spectrometry a peptide consisting of the amino acid sequence of any one of SEQ ID Nos 2, 10-42 and 44.

Embodiment 6. The method of embodiment 4 or 5, wherein the method comprises: contacting the sample comprising or suspected of comprising BoNT/A with antibodies specific for conserved heavy and/or antibodies specific for conserved light chain sequences among BoNT/A subtypes, wherein the antibodies specific for conserved heavy and/or the antibodies specific for conserved light chain sequences are coupled to solid supports, to form a solid support-antibody-heavy chain complex and/or a solid support-antibody-light chain complex;

separating the solid support-antibody-heavy and/or solid support-antibody-light chain complexes from other substances present in the sample;

digesting the heavy and light chains with trypsin to create trypsin fragments of the heavy and light chains;

separating solid support and antibodies from the trypsin fragments; and

subjecting the trypsin fragments to mass spectrometry, in particular liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS) to determine the sequences of the fragments.

Embodiment 7. The method of any one of embodiments 1 to 6, wherein the mass spectrometry is liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS).

Embodiment 8. The method of any one of embodiments 1 to 7, further comprising:
5 comparing the sequence of the proteolytic fragments to known sequences of BoNT/A subtype sequences to determine the subtype present in the sample.

Embodiment 9. The method of any one of embodiments 1 to 8, wherein the antibody binds to a peptide selected from the group consisting of the amino acid sequences QVPVSYDSTYLSTDNEK (SEQ ID NO:3), DTFTNPEEGDLNPPPEAK (SEQ ID NO:4) and
10 GPLGSSTDIPFQLSKYVDNQRLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN CMENNSGWKVS LNYGEIHWTLQDTQEIKQRVVFYKYSQMINISDYINRWIFVTITNRLNN SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL YDNQSN SGILKDFWGDYLQYDKPYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV
15 MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47).

Embodiment 10. The method of any one of embodiments 1 to 9, wherein the method is a diagnostic method to determine what BoNT/A subtype is present in the sample.

Embodiment 11. The method of any one of embodiments 1 to 10, wherein the
20 sample does not include viable neurotoxin-producing bacteria or DNA from the *C. botulinum* subtype.

Embodiment 12. The method of any one of embodiments 1 to 11, wherein the
25 sample comprises a foodstuff sample, water, a sample from a manufactured cosmetic or pharmaceutical product, an environmental, or biological sample.

Embodiment 13. The method for quantitatively determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising adding an internal isotypically stable standard to the sample to be analyzed, performing the steps of any one of embodiments 1 to 12 and quantitating the BoNT/A subtype.

Embodiment 14. An antibody that specifically binds to a peptide consisting of the
30 amino acid sequence QVPVSYDSTYLSTDNEK (SEQ ID NO:3) or DTFTNPEEGDLNPPPEAK (SEQ ID NO:4) or

GPLGSSTDIPFQLSKYVDNQRLLSFTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK
 VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
 CMENNSGWKVSLSNYGEIHWTLQDTQEIKQRVVFVKYSQMINISDYINRWIFVTITNNRLNN
 SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL
 5 YDNQSN SGILKDFWGDYLYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV
 MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
 EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL
 VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47), wherein the
 antibody is bound to a solid support.

10 Embodiment 15. The antibody of embodiment 14, wherein the antibody is a polyclonal antibody.

 Embodiment 16. The antibody of embodiment 14, wherein the antibody is a monoclonal antibody.

 Embodiment 17. The antibody of any one of embodiments 14 to 16, wherein the
 15 antibody is a rabbit antibody.

 Embodiment 18. A kit for determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, the kit comprising an antibody that specifically binds to a peptide selected from the group consisting of the amino acid sequences QVPVSYDSTYLSTDNEK (SEQ ID NO:3), DTFTNPEEGDLNPPPEAK (SEQ ID NO:4) and
 20 GPLGSSTDIPFQLSKYVDNQRLLSFTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK
 VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
 CMENNSGWKVSLSNYGEIHWTLQDTQEIKQRVVFVKYSQMINISDYINRWIFVTITNNRLNN
 SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL
 YDNQSN SGILKDFWGDYLYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV
 25 MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
 EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL
 VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47).

 Embodiment 19. The kit of embodiment 18, further comprising a solid support for binding to the antibody of the kit.

30 Embodiment 20. The kit of embodiment 19, wherein the solid support is a bead.

 Embodiment 21. The kit of embodiment 20, wherein the bead is a magnetic bead.

Embodiment 22. Use of a method according to any one of embodiments 1 to 11, to determine the presence or the quantity of BoNT/A or of a particular subtype thereof present as active ingredient in a cosmetic or a pharmaceutical product.

5

EXAMPLES

The invention will be further explained by the following Examples, which are intended to be purely exemplary of the invention, and should not be considered as limiting the invention in any way.

Example 1: Materials, Chemicals, and Experimental Procedures

10

Animals: BALB/C mice (female, 4 weeks old) were purchased from Charles River. The animals were fed with a standard diet and water ad libidum. Any mice found to be severely injured were euthanized. Determination of mouse lethal dose (MLD₁₀₀) for *C. botulinum* toxin was performed as previously described (32). Briefly, 0.5 ml of serial 10- fold and then 2-fold dilutions of toxin samples in phosphate buffer (50 mM, pH 6.3) containing 0.2% gelatin were injected intraperitoneally (3 mice per dilution). The mice were observed up to 4 days. Mice surviving four days were considered to have survived.

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Cells, Peptides, and Toxin Assays:

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C. botulinum strains used in this study are listed in Table 1. *C. botulinum* strains were grown in trypticase-glucose-yeast extract medium in anaerobic conditions for four days. Culture supernatants were stored at -80°C until use. The presence of toxin in the supernatant was first detected by an immune-chromatographic test (BTA Gold Assay; NBC-Sys, France) and the toxin activity (MDL₁₀₀ ml⁻¹) was then tested by mouse bioassay as previously described (31).

25

All of the peptides used were provided by Proteogenix (France) with a purity of greater than 95%. The lyophilized peptides received from the supplier were suspended in H₂O/HPLC-grade acetonitrile (ACN) 50/50 at a concentration of 1 mg ml⁻¹ and stored at -20°C. Rabbit anti-peptides polyclonal antibody (anti-peptides PAb) was specifically generated by Proteogenix for the immunomagnetic separation. Two peptides, Q67-K84 (QVPVSYDSTYLSTDNEK (SEQ ID NO:3)) and D49-K66 (DTFTNPEEGDLNPPPEAK (SEQ ID NO:4)) from the light chain (Lc) were selected for the immunization. The peptide Q67-K84 is common to the subtypes A1, A2, A3, A5, A6, A7, and A8. The peptide D49-K66 is present in all subtypes, but shows one mutation in the A7 subtype (I50 to T) and in the A8 subtype (K55 to E). Despite these mutations, the two peptides were recognized by the anti-peptides PAb. The reactivity of the anti-

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peptides PAb with the BoNT/A1, /A2, /A3, /A5, /A7, and /A8 was checked using a standard ELISA procedure. The absence of cross-reactivity against BoNT/A heavy chain (Hc), BoNT/B and BoNT/E was tested by Western blot and no cross-reactivity was detected.

5 **Table 1:** List of *C. botulinum* strains used in this study, their neurotoxin gene cluster, and mouse lethal activity of culture supernatant as tested with mouse bioassay

Neurotoxin Gene Cluster	Strain	Lethal Activity (MLD100/ml)
<i>ha-bont/A1</i>	Hall	2×10^4
<i>ha-bont/A1</i>	Legroux	1×10^5
<i>orfX-bont/A1(B)</i>	NCTC2916	1×10^5
<i>orfX-bont/A1</i>	200.04	2×10^5
<i>orfX-bont/A2</i>	136.06	2×10^4
<i>orfX-bont/A2</i>	133.06	2×10^4
<i>orfX-bont/A2</i>	9336	2×10^5
<i>orfX-bont/A2</i>	181.02	2×10^5
<i>orfX-bont/A2</i>	1618	2×10^5
<i>orfX-bont/A3</i>	Loch Maree	2×10^3
<i>ha-bont/A5</i>	126.07	2×10^4
<i>orfX-bont/A7</i>	148.08	2×10^4
<i>orfX-bont/A7</i>	217.12	1.6×10^4

The food and environmental samples were from a local source (local tap water), local
 10 grocery (orange juice), and French Defense Health Service (human serum).

Preparation of Anti-peptides PAb Coated Magnetic Beads:

Protein G-conjugated magnetic beads (Dynabeads Protein G, Novex) (250 µl) were transferred in a 2 ml Eppendorf low binding microcentrifuge tube and were washed three times with 500 µl of PBST (Phosphate Buffer Salt, 0.1 % Tween 20). After removing the final solution wash, the beads were suspended in 500 µl of PBST containing 100 µg ml⁻¹ anti-peptides PAb. The mixture was incubated for 30 min with rotation at laboratory temperature. The beads were washed three times with 1 ml of PBS containing 0.15 M NaCl. Antibodies were cross-linked to the beads by incubating for 30 min with rotation at laboratory temperature in 1250 µl of PBS containing 0.15 M NaCl and 5 mM BS₃ (crosslinking reagent). 65 µl of Tris/HCl (1 M, pH 7.5) were added for quenching the cross-linking reaction. After incubation for 15 min at laboratory temperature with rotation, the beads were washed three times with 1 ml of PBST and resuspended in 250 µl of PBST. They were stored at 4°C for as long as 3 weeks.

In-gel Digestion for LC-ESI-MS/MS Analysis:

Each culture supernatant of distinct *C. botulinum* strains from BoNT subtypes was precipitated with 60% of ammonium sulfate. After precipitation, the pellet was dissolved in 50 mM sodium citrate buffer, pH 5.5, and dialyzed overnight at 4°C against the same buffer. The dialyzed sample was fractionated via molecular size chromatography (33) using an AKTÄ system and an S300 column (HiPrep 26/60 Sephacryl S-300 HR; GE Healthcare Bio-Sciences). For each subtype, the relevant fractions were pooled and considered as pre-purified complexes. The amount of proteins on pre-purified complexes was determined by the Bio-Rad protein assay with Bovine IgG as a standard protein. 1 to 2 µg of each pre-purified complex for each BoNT subtype were run on a SDS-PAGE. After migration, gels were fixed and stained with Coomassie staining solution (Bio-Rad, Marnes la Coquette, France). Based on protein size, gel regions containing protein to identify were excised and cut into small pieces. Gel pieces were destained by washing cycles in water and acetonitrile for 10 min. After destaining, gel pieces were dried by vacuum centrifugation and reduced with DTT (10mM) in 50 mM ammonium carbonate buffer and alkylated with iodoacetamide (55mM) in 50 mM ammonium carbonate buffer. Gel pieces were washed again with 50 mM ammonium carbonate buffer and dehydrated in 100% acetonitrile (ACN) before trypsin digestion (15 µg ml⁻¹) at 4°C for 45 min and 2 hours at 37°C. Tryptic peptides were extracted from the gel in three successive steps (15 min each), using 2 cycles of 5% formic acid, and then 100% ACN extraction solutions. After drying by vacuum

centrifugation, tryptic peptides were resuspended in 20 µl of 1% TFA before LC-ESI-MS/MS analysis.

LC-ESI-MS/MS Analysis on an Ion Trap Mass Spectrometer:

Liquid Chromatography-Electrospray Ionization-Multi-Stage/Mass Spectrometry (LC-ESI-MS/MS) analysis was performed on an Esquire 6000 mass spectrometer (ion trap mass spectrometer; Bruker Daltonics, Bremen, Germany) connected with an Ultimate 3000 high performance liquid chromatograph (Dionex Corp.; Sunnyvale, CA). The protein digest (10 µl) was injected on a 0.3 × 150 mm PepMap100 capillary column, particle size 5 µm, 100 Å pore size (LC Packings; Amsterdam, The Netherlands). Samples were eluted using a gradient starting with a linear increase from 5 to 10% acetonitrile over 4 min, followed by an increase from 10 to 80% acetonitrile over 42 min with 0.2% acid formic throughout as additives. The data acquisition was performed using the data-dependent mode where the three highest intensity precursors in an MS1 survey scan were selected for collision-induced dissociation. The resulting MS/MS data were searched for protein candidates with a database search against NCBI nr database using MASCOT software (Matrix version 2.2.07, Sciences, London, U.K.). The mass tolerance of precursor ions and fragment ions was 0.5 Da. Resulting peptides were filtered with a significance threshold of $p < 0.05$.

Immunocapture Enrichment and Trypsin Digestion for LC-QqQ-MS/MS Analysis:

Culture supernatants or samples, crude or diluted in PBS, in a volume of 500 µl were treated with beta-mercaptoethanol (35 µl of 14 M stock solution) for 10 min at 95°C in a dry heating block. After cooling at room temperature, each sample was mixed with 100 µl of PAb-coated magnetic beads in an Eppendorf low binding microcentrifuge tube. The mixture was incubated for 120 min at laboratory temperature with rotation. The beads were then recovered using a magnetic particle concentrator and washed three times with 1 ml of HEPES buffer (pH 7.6, 50 mM). The beads were then suspended in 100 µl of 100 mM ammonium carbonate buffer (pH 8.5) and incubated for 10 min at 95°C. 15 µl of 100 µg ml⁻¹ aqueous trypsin solution (sequencing grade modified trypsin, Promega) was added and the enzymatic digestion was carried out at 38°C for 120 min. After digestion, the magnetic beads were removed and 2 µl of analytical grade formic acid (FA) was added. The solution was vacuum-dried and resuspended in 50 µl of H₂O/ ACN/FA 93/5/2 before the LC-QqQ-MS/MS analysis in MRM mode.

LC-QqQ-MS/MS Analysis in MRM Mode:

Liquid Chromatography-triple quadrupole mass spectrometry-Multi-Stage/Mass Spectrometry (LC-QqQ-MS/MS) was used to analyze samples. The HPLC system used for this analysis was an Ultimate 3000 RSLC equipped with a degasser, a binary pump, an auto-injector and a column oven (Thermo Scientific) coupled to a TSQ Quantiva electrospray ionization triple quadrupole mass spectrometer (Thermo Scientific). Chromatographic separations were performed on Zorbax SB-C18 2.1 × 150 mm, 3.5 µm, 80Å pore size (Agilent; Santa Clara, CA) using 0.2% (v/v) formic acid as eluent A and acetonitrile with 0.2% formic acid as eluent B with a flow rate of 200 µl/min at 25°C. Samples (10 µl) were analyzed in a gradient mode: 5% eluent B (0–2 min) and 50% B (30 min). The mass spectrometer ESI source was operated in positive ionization mode. The following conditions were applied: spray voltage, 4000 V; sheath gas, 50 Arb; aux gas, 10 Arb; sweep gas, 5 Arb; ion transfer tube temp, 325°C; vaporizer temp, 275°C; CID gas, argon, 2 mTorr.

Food and Biological Samples:

Food and human serum samples were artificially spiked with culture supernatant of each *C. botulinum* A subtype, and 0.5 ml of each sample were treated as above described for the culture supernatants except that the serum samples were not heated. Serial 10 fold dilutions of each sample were tested by mouse bioassay and by immunocapture/LC-QqQ-MS/MS in MRM mode.

Example 2: Selection of Specific Peptides for Botulinum Toxin A Subtype Detection

MS-based analysis can distinguish proteins on the basis of amino acid sequence. BoNT/A1 to BoNT/A8 subtypes share 84.5 to 97.2% identity at the amino acid level (see Table 2).

Table 2: Amino acid sequence identity (%) of BoNT/A subtypes. Amino acid sequences from representative strains (and Genbank Accession No.) are: BoNT/A1 from strain Hall (YP_001386738.1); BoNT/A2 from strain Kyoto (CAA51824.1); BoNT/A3 from strain Loch Maree (YP_0017157303.1); BoNT/A4 from strain BA657 (YP_002860313.1); BoNT/A5 from strain IBCA94-0216 (ACT33194.1); BoNT/A6 from strain CDC 41370 (ACW83608.1); BoNT/A7 from strain 148.08 (AFV13854.1); and BoNT/A8 from strain 217.12 (AHA83316.1).

	A2	A3	A4	A5	A6	A7	A8
A1	90.0	84.9	89.4	97.2	95.7	93.8	93.4
A2		93.4	88.4	90.5	91.7	89.9	93.5
A3			84.5	85.4	86.5	85.1	88.0
A4				87.6	87.9	86.9	89.1
A5					95.9	94.4	93.7
A6						93.0	93.1
A7							91.4

The inventors first identified the tryptic cleavage sites on the sequences of the Lc chain (amino acids 1 to 449) of each BoNT/A subtype and the inventors then analyzed all the subsequent predicted peptides for their specificity versus the other BoNT/A subtypes by Blast (NCBI). Thereafter, 26 peptides of each BoNT/A subtypes were analyzed *in silico*. Two to 12 unique peptides for each BoNT/A subtype were synthesized and analyzed in MS full scan and MS/MS to identify predominant product ions (see Table 3).

Table 3: Proteolytic Peptides (peptide size between 7 and 25 amino acids) of BoNT/A1 to A8 Light Chains

Specificity	Tryptic Digest Fragment	Peptide Sequence	SEQ ID NO
Conserved ^a	I24-K34	IPNAGQMGPVK	(SEQ ID NO:5)
Conserved ^a	Q67-K84	QVPVSYDSTYSTDNEK	(SEQ ID NO:6)
Conserved ^a	G114-K128	GIPFWGGSTIDTELK	(SEQ ID NO:7)
Conserved ^b	N178-R187	NGYGSTQYIR	(SEQ ID NO:8)
Conserved ^a	L232-R241	LYGLAINPNR	(SEQ ID NO:1)

Conserved ^b	N418-K427, N414-K423 for BoNT/A3	NFTGLFEFYK	(SEQ ID NO:9)
BoNT/A1 and A6 ^c	S167-R177	SFGHEVLNLTR	(SEQ ID NO:10)
BoNT/A1 and A6 ^c	V382-R393	VNYTIYDGFNLR	(SEQ ID NO:11)
BoNT/A2 and A8 ^d	F213-R231	FATDPAVTLAHELIHAEHR	(SEQ ID NO:12)
BoNT/A2	D292-K299	DVASTLNK	(SEQ ID NO:2)
BoNT/A2 and A7 ^c	S302-K314	SIIGTTASLQYMK	(SEQ ID NO:13)
BoNT/A2	M344-K359	MLTEIYTEDNFVNFFK	(SEQ ID NO:14)
BoNT/A2 and A8 ^d	I376-K387	INIVPDENYTIK	(SEQ ID NO:15)
BoNT/A2	G394-R411	GANLSTNFGNQNTINSR	(SEQ ID NO:16)
BoNT/A3	I38-R48	IHEGVWVIPER	(SEQ ID NO:17)
BoNT/A3	I98-R105	IYSTGLGR	(SEQ ID NO:18)
BoNT/A3	M106-K113	MLLSFIVK	(SEQ ID NO:19)
BoNT/A3	V129-R145	VIDTNCINVIEPGGSYR	(SEQ ID NO:20)
BoNT/A3	S146-K166	SEELNLVITGPSADIIQFECK	(SEQ ID NO:21)
BoNT/A3	S167-R177	SFGHDVFNLTR	(SEQ ID NO:22)
BoNT/A3	T247-R264	TNAYYEMSGLEVSFEELR	(SEQ ID NO:23)
BoNT/A3	T265-R280	TFGGNDTNFIDSLWQK	(SEQ ID NO:24)
BoNT/A3	D285-R295	DAYDNLQNIAR	(SEQ ID NO:25)
BoNT/A3	T302-K314	TIVGTTTPLQYMK	(SEQ ID NO:26)
BoNT/A3	Y321-K330	YFLSEDASGK	(SEQ ID NO:27)
BoNT/A3	G348-K359	GFTELEFVNPFK	(SEQ ID NO:28)

BoNT/A5	I98-R105	IYSELGR	(SEQ ID NO:29)
BoNT/A5	T265-K272	TFGEHDAK	(SEQ ID NO:30)
BoNT/A5	I376-R393	INIVPEVNYTIYDGFNLR	(SEQ ID NO:31)
BoNT/A6	N394-K415	NTNLAANFNGQNTTEINNMNFAK	(SEQ ID NO:32)
BoNT/A7	D49-K66	DIFTNPEEGDLNPPPEAK	(SEQ ID NO:33)
BoNT/A7	S146-K166	SEELNLVIIGPSADIINFECK	(SEQ ID NO:34)
BoNT/A7	F213-R231	FAIDPAVTLAHELIHAGHR	(SEQ ID NO:35)
BoNT/A7	E292-K299	EVASILNK	(SEQ ID NO:36)
BoNT/A7	M376-R393	MNIVPEVNYTIYDGFNLR	(SEQ ID NO:37)
BoNT/A8	D12-K23	DTVNGVDIAYIK	(SEQ ID NO:38)
BoNT/A8	D49-K55	DTFTNPK	(SEQ ID NO:39)
BoNT/A8	E56-K66	EGDLNPPPEAK	(SEQ ID NO:40)
BoNT/A8	T265-K272	TFGGHNAK	(SEQ ID NO:41)
BoNT/A8	N394-R411	NTNLAANFNGQNTTEINSR	(SEQ ID NO:42)

^a Peptides conserved in BoNT/A1-/A3 and BoNT/A5-/A8 light chains

^b Peptides conserved in BoNT/A1 to A8 light chains

^c Peptides common to BoNT/A1 and BoNT/A6 light chains

^d Peptides common to BoNT/A2 and BoNT/A8 light chains

5 ^e Peptides common to BoNT/A2 and BoNT/A7 light chains

10 Then, they were analyzed in LC-QqQ-MS/MS in MRM mode, and for each subtype, two specific peptides yielding the best response were selected. One peptide was common to both BoNT/A2 and BoNT/A8, and two other to BoNT/A1 and BoNT/A6. Moreover, two additional peptides, which were conserved in all BoNT/A subtypes, were also selected (see Table 4).

Table 4: Synthetic peptides specific for each light chain BoNT/A subtype and MRM transitions. Three transitions for each analysis, n=3.

Specificity	Tryptic Digest Fragment	Proteolytic Peptide Sequence	SEQ ID NO	SRM Transitions Precursor (m/z)	SRM Transitions Product (m/z)	Retention Time (min.)
Conserved	G114-K128	GIPFWGGSTIDTELK	7	810.9	601.3 (b5) 725.9 (y13) 1020.5 (y10)	20.5
Conserved	L232-R241	LYGIAINPNR	1	565.8	334.2 (b3) 684.4 (y6) 854.5 (y8)	15.7
BoNT/A1	S167-R177	SFGHEVLNLTR	10	424.9	389.3 (y3) 503.3 (y4) 657.3 (b6)	15.3
BoNT/A1	V382-R393	VNYTIYDGFNLR	11	737.9	214.1 (b2) 884.4 (y7) 997.5 (y8)	19.0
BoNT/A2	D292-K299	DVASTLNK	2	424.2	187.1 (b4) 215.1 (b2) 633.4 (y6)	10.1
BoNT/A2 and /A8	I376-K387	INIVPDENYTIK	15	709.9	228.1 (b2) 341.2 (b3) 979.5 (y8)	16.3
BoNT/A3	S167-R177	SFGHDVFNLTR	22	431.6	276.2 (y2) 389.3 (y3) 503.3 (y4)	16.4
BoNT/A3	G348-K359	GFTELEFVNPFK	28	714.4	391.2 (y2) 505.3 (y3) 880.5 (y7)	22.0

BoNT/A5	I98-R105	IYSELGR	29	469.8	277.2 (b2) 662.4 (y6) 825.4 (y7)	12.1
BoNT/A5	I376-R393	INIVPEVNYTIYDGFNLR	31	714.0	440.3 (b4) 721.4 (y6) 884.4 (y7)	22.6
BoNT/A7	E292-K299	EVASILNK	36	437.3	487.3 (y4) 574.4 (y5) 645.4 (y6)	13.1
BoNT/A7	M376-R393	MNIVPEVNYTIYDGFNLR	37	720.0	359.17 (b3) 458.2 (b4) 884.4 (y7)	22.0
BoNT/A8	E56-K66	EGDLNPPPEAK	40	583.8	541.3 (y5) 638.4 (y6) 752.4 (y7)	10.5

For each BoNT/A subtype, two conserved specific peptides were first identified. For BoNT/A subtype comprising common specific peptides (*e.g.* peptide I376-K387 (SEQ ID NO:15) common to BoNT/A2 and BoNT/A8), it was necessary to detect the two specific peptides to determine the BoNT/A subtype (*e.g.* peptide D292-K299 (SEQ ID NO:2) and peptide I376-K387 (SEQ ID NO:15) to determine the BoNT/A2 subtype). For BoNT/A subtype not comprising common specific peptides with any other BoNT/A subtype, only one specific peptide had be detected to determine the BoNT/A subtype (*e.g.* peptide S167-R177 (SEQ ID NO:10) or peptide V382-R393 (SEQ ID NO:11) to determine the BoNT/A1 subtype), or, as an additional control, the two specific peptides could be detected to determine the BoNT/A subtype (*e.g.* peptide S167-R177 (SEQ ID NO:10) and peptide V382-R393 (SEQ ID NO:11) to determine the BoNT/A1 subtype).

The transition values allowed the identification of a unique peptide sequence specific to a BoNT/A subtype. The above-mentioned retention times were obtained using the previously described experimental conditions, and the above-mentioned values for the retention times would be different if the skilled person would use other experimental conditions (*e.g.* another type of column, a different gradient, ...).

Following fragmentation (*e.g.* Collision-induced dissociation (CID), Electron-capture dissociation (ECD)), the obtained fragments are named “product ions” (newly formed ions) as opposed to the “precursor ions” corresponding to ions coming from the ionization of the peptide. When fragmentation occurs at the level of the peptide bond (*e.g.* Collision-induced dissociation (CID)), the ion containing the N-terminal part of the fragmented peptide is called ion b, whereas the ion containing the C-terminal part is called ion y. In triple quadrupole, peptides are advantageously detected according to the ratio m/z of the precursor ions and the product ions (called “transitions”) (see Figure 4).

Example 3: Identification of BoNT/A Subtype in Culture Supernatants

To identify BoNT/A subtypes in a complex environment like culture supernatant, the inventors used an immunocapture step. For that purpose, magnetic beads were coated with antibodies raised against two synthetic peptides conserved in BoNT/A light chain subtypes as described above. Culture supernatant samples (500 μ l) were reduced by addition of beta-mercaptoethanol to allow the separation of L and H chains and were then incubated with coated magnetic beads. The procedure described above was followed from here. It is noteworthy that beta-mercaptoethanol facilitated the Lc chain capture, since experiments without beta-mercaptoethanol pre-treatment resulted in lower detection sensitivity. After washing, the bound material was digested by trypsin *in-situ*, dried, and analyzed by LC-MS/MS. An example of conserved peptide detection by LC-MS/MS in culture supernatants of representative strains of each BoNT/A subtype is shown in Fig. 1. *Clostridium sporogenes*, which is closely related to *C. botulinum* A strains, showed no detectable peptide corresponding to L chain fragment.

Fig. 2 shows identification of BoNT/A2 through detection of the specific subtype peptide DVASTLNK (SEQ ID NO:14) by LC-MS/MS in *C. botulinum* culture supernatants. The global results of peptide detection in *C. botulinum* culture supernatants are listed in Table 5.

Table 5: Results of peptide detection by LC-QqQ-MS/MS in MRM mode in *C. botulinum* culture supernatants. n=3.

	L232 - R241 Cons	G114 - K128 Cons	S167- R177 BoNT /A1	V382- R393 BoNT /A1	D292- K299 BoNT /A2	I376- K387 BoNT/ A2/A8	S167- R177 BoNT /A3	G348- K359 BoNT /A3	I98- R105 BoNT /A5	I376- R393 BoNT /A5	E292- K299 BoNT/ A7	M376- R393 BoNT/ A7	E56- K66 BoNT/ A8
Hall - BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
Legroux BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
NCTC29 16	+	+	+	+	-	-	-	-	-	-	-	-	-
200.04 - BoNT/A1	+	+	+	+	-	-	-	-	-	-	-	-	-
136.06 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
133.06 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
9336 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
181.02 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
1618 - BoNT/A2	+	+	-	-	+	+	-	-	-	-	-	-	-
Loch Maree BoNT/A3	+	+	-	-	-	-	+	+	-	-	-	-	-
126.07 - BoNT/A5	+	+	-	-	-	-	-	-	+	+	-	-	-
148.08 - BoNT/A7	+	+	-	-	-	-	-	-	-	-	+	+	-
217.12 - BoNT/A8	+	+	-	-	-	+	-	-	-	-	-	-	+

To validate the selection of these peptides for the identification of BoNT/A subtypes, BoNT preparations of each subtype (1 to 2 µg) were run on a SDS-PAGE and the bands containing the Lc were cut out of the gel. After in-gel trypsin digestion as described above, the peptides were extracted and analyzed with LC-ESI-MS/MS. BoNT/A4 and BoNT/A6 were not included in this study due to the difficulty of obtaining BoNT/A4 preparations containing sufficient amounts of toxin and to the fact that BoNT/A6 (strain CDC41370) was not available in our collection. However, it is to be understood that they are equally amenable to the procedure as the other subtypes.

The selected conserved peptides were detected in all the *C. botulinum* culture supernatants tested, whereas the selected specific subtype peptides were only identified in the corresponding *C. botulinum* subtype culture supernatants. However, the I376-K387 peptide was

detected in both *C. botulinum* A2 and A8 subtype culture supernatants, in contrast to the D292-K299 peptide which was only found in *C. botulinum* A2 strains (see Table 5).

Example 4: Sensitivity

The limit of detection (LOD) for subtyping BoNT/A by the LC-QqQ-MS/MS in MRM mode was estimated by using serial dilutions of *C. botulinum* culture supernatants. LOD corresponded to the highest dilution giving a signal with a signal-to-noise ratio of 3. The results with representative *C. botulinum* culture supernatant subtypes and expressed in equivalent MLD₁₀₀ which have been determined by the mouse bioassay, are shown in Table 6. The MS methodology could detect BoNT ranging from 20 to 140 MLD/0.5 ml according to the subtype.

Table 6: Estimated limit of detection (LOD). LOD is expressed in equivalent MLD₁₀₀/0.5 ml. Signal to noise ratio of 3. Results are mean values $\pm$ SD. N+3.

<i>C. botulinum</i> A Subtype	Detected Peptide	LOD (LMLD ₁₀₀)
Hall (A1)	S167-R177	110 $\pm$ 16
9336 (A2)	D292-K299	30 $\pm$ 4.5
Loch Maree (A3)	S167-R177	50 $\pm$ 7.5
126.07 (A5)	I98-R105	20 $\pm$ 3
148.08 (A7)	M376-R393	140 $\pm$ 21
271.12 (A8)	E56-K66	50 $\pm$ 7

Example 5: Identification of BoNT/A Subtype in Different Matrices

In order to validate the method of LC-QqQ-MS/MS in MRM mode with biological, food, or environmental samples, LOD was determined in various representative matrices (tap water, orange juice, and human serum) spiked with the distinct BoNT/A subtypes. As shown in Table 7, the BoNT/A subtypes A1 to A8 were successfully identified in all the different samples.

Using non-inoculated TGY medium as background, BoNT peptides were easily identified at the

LOD as shown in Fig. 3 for BoNT/A2. Note that no interfering peak at the expected retention times was observed in the tap water and orange juice matrices. The best LOD values were obtained with tap water and orange juice, and were comparable to those obtained with culture supernatants (see Table 6), whereas the detection was slightly lower (4 to 10 fold less) in human serum due to a higher background.

Table 7: Estimated limit of detection (LOD expressed in equivalent MLD₁₀₀ /0.5 ml) in various samples. Signal to noise ratio of 3. n=3.

Matrices	Hall (A1)	9336 (A2)	Loch Maree (A3)	126.07 (A5)	148.08 (A7)	271.12 (A8)
Tap Water	70	20	50	15	110	50
Orange Juice	110	50	70	20	150	60
Human Serum	300	200	250	100	500	300

Example 6: Method of quantification of BoNTs in complex matrices by mass spectrometry

A calibration range was carried out using a variable quantity of the BoNT/A1 light chain and a known quantity (internal standard) of the BoNT/A1 light chain labeled with stable isotopes such as ¹³C₆ on lysine and arginine amino acid residues. The labeled light chain of BoNT/A1 (labeled at a rate higher than 95%) was prepared *via* a classical method well-known to the skilled person, using a prototrophic bacterial strain. Both types of light chains were added in a matrix (TGY medium). The obtained samples were heated in the presence of betamercaptoethanol and immunocaptured using antibodies specific for the two peptides Q67K84 (SEQ ID NO:3) and D49K66 (SEQ ID NO:4) as previously described. These two peptides were chosen due to the fact that they were common to A1 to A3 and A5 to A8 subtypes, their use allowing the quantification of all BoNT/A subtypes (except A4 that was not taken into account in this experiment). Ratios of the intensity (or area) of the peptide L232R241 (SEQ ID

NO:1)/L232[¹³C₆]R241 or L232R241* (SEQ ID NO:45) and G114K128 (SEQ ID NO:7)/G114[¹³C₆]K128 or G114K128* (SEQ ID NO:46) on the three transitions were calculated for each concentration of the BoNT/A1 light chain. The calibration range was carried out using different quantities of the BoNT/A1 light chains, taking into account the sample preparation steps (immunocapture, *e.g.* on beads). A linear signal was obtained for quantities of up to 6500 ng of light chain in the sample (linear regression coefficient > 0.995) (see Figure 5). Quantities higher than 6500 ng (equivalent to 19.5 µg of BoNT) have not been tested due to the fact that these quantities were not representative of the expected contamination of the sample. Figure 5 showed the good linearity of the method for the three transitions of the two peptides L232R241 (SEQ ID NO:1) and G114K128 (SEQ ID NO:7).

Botulinum neurotoxins (BoNTs) of different subtypes were quantified in culture supernatants of *C. botulinum* against a calibration range consisting of variable quantities of the BoNT/A1 light chain (LcA1) and a constant quantity of the labeled BoNT/A1 light chain (LcA1*). The range was carried out for each series of analyses and the supernatants were optionally diluted so that values located within the limits of quantification of the range were obtained. All the prepared samples were reduced, immunocaptured using antibodies specific for the peptides Q67K84 (SEQ ID NO:3) and D49K66 (SEQ ID NO:4) and digested *in situ*, *e.g.* with trypsin, using above described protocols. After analyses of the samples by mass spectrometry, preferably liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS) as above described, the obtained results were used for calculation of the areas for each transition using the Xcalibur™ software (Thermo Fisher Scientific). Crude data were then uploaded to the Skyline™ software (MacCoss Lab) to determine for each sample (internal standard and sample to be quantified) the ratios of the areas of the three transitions for each unlabeled peptide (L232R241 (SEQ ID NO:1) and G114K128 (SEQ ID NO:7)) over the areas of the three transitions for each labeled peptide (L232[¹³C₆]R241 or L232R241* (SEQ ID NO:45) and G114[¹³C₆]K128 or G114K128* (SEQ ID NO:46)). The equation $f(\text{quantities LcA1}) = \text{Ratio}_{[\text{LcA1}/\text{LcA1}^*]}$ was plotted. The quantity of light chain present in the sample was calculated according to the obtained calibration range and converted to the quantity of neurotoxin according to the stoichiometry (neurotoxin = 3 x LcA1).

Quantification of the LcA1 in culture supernatants

The BoNT/A1 light chain present in the neurotoxin and in the purified botulinum complex (both commercialized by Metabionics) was quantified as well as the BoNT/A1, /A2, /A3, /A5, /A7 and /A8 light chains from the culture supernatants. Each sample was quantified at different dilutions against a range of LcA1 and LcA1* (see Table 8).

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Table 8: Results of quantification of the light chain in the samples. ^{a)} Two independent productions of supernatant *C. botulinum* A3 strain Loch Maree were carried out (supernatant 1 and supernatant 2). ^{b)} Quantity of neurotoxin is obtained by multiplying by 3 the quantity of measured light chain.

Sample	Subtype	Dilution	Ratio LcA1/LcA1*	Measured LcA (ng)	Quantity of measured average LcA (ng)	Quantity of determined neurotoxin ^(b) (ng)
Neurotoxin Metabionics	BoNT/A1	1/1000	1.6	500.10 ³	415.10 ³	1245.10 ³
Neurotoxin Metabionics	BoNT/A1	1/2000	0.65	330.10 ³		
Complex Metabionics	BoNT/A1	1/250	1.05	80.10 ³	87.10 ³	260.10 ³
Complex Metabionics	BoNT/A1	1/500	0.756	95.10 ³		
Supernatant <i>C. bot.</i> strain Hall	BoNT/A1	1	0.145	22	28.6	86
Supernatant <i>C. bot.</i> strain Hall	BoNT/A1	1/2	0.100	30		
Supernatant <i>C. bot.</i> strain Hall	BoNT/A1	1/1	0.211	33		
Supernatant <i>C. bot.</i> strain Hall	BoNT/A1	1/1	0.1645	26		
Supernatant <i>C. bot.</i> strain Hall	BoNT/A1	1/1	0.202	32		
Supernatant <i>C. bot.</i> strain 9336	BoNT/A2	1/40	0.152	937	1210	3630
Supernatant <i>C. bot.</i> strain 9336	BoNT/A2	1/50	0.165	1278		

Supernatant <i>C. bot.</i> strain 9336	BoNT/A2	1/100	0.091	1327		
Supernatant <i>C. bot.</i> strain 9336	BoNT/A2	1/50	0.189	1483		
Supernatant <i>C. bot.</i> strain 9336	BoNT/A2	1/10	0.625	1023		
Supernatant 1 <i>C. bot.</i> strain Loch Marce	BoNT/A3	1/2	6.801	2263	2118	6354
Supernatant 1 <i>C. bot.</i> strain Loch Marce	BoNT/A3	1/5	2.938	2439		
Supernatant 1 ^(a) <i>C. bot.</i> strain Loch Marce	BoNT/A3	1/10	1.002	1651		
Supernatant 2 ^(a) <i>C. bot.</i> strain Loch Marce	BoNT/A3	1/5	0.280	224	224	672
Supernatant <i>C. bot.</i> strain 126.07	BoNT/A5	1/5	0.3	240	282	845
Supernatant <i>C. bot.</i> strain 126.07	BoNT/A5	1/5	0.31	249		
Supernatant <i>C. bot.</i> strain 126.07	BoNT/A5	1/5	0.399	323		
Supernatant <i>C. bot.</i> strain 126.07	BoNT/A5	1/10	0.200	314		
Supernatant <i>C. bot.</i> strain 148.08	BoNT/A7	1/1	0.204	32	25	75
Supernatant <i>C. bot.</i> strain 148.08	BoNT/A7	1/1	0.131	20		
Supernatant <i>C. bot.</i> strain 148.08	BoNT/A7	1/1	0.152	23		

Supernatant <i>C. bot.</i> strain 217.12	BoNT/A8	1/1	0.226	36	32	96
Supernatant <i>C. bot.</i> strain 217.12	BoNT/A8	1/1	0.198	31		
Supernatant <i>C. bot.</i> strain 217.12	BoNT/A8	1/1	0.189	30		

Determination of the specific activity for BoNT/A subtypes

The specific activity for BoNT/A was calculated according to the level of neurotoxin present in the quantified samples associated to its lethal activity (See Table 9).

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Table 9: Determination of specific activities for BoNT/A subtypes.

Sample and strain	Quantity of measured Lc (ng)	Quantity of calculated neurotoxin (ng)	Lethal doses from the dosed sample in MDL (E = 500 μ L)	Specific activity (pg/MDL)	Relative standard deviation RSD (%)
Neurotoxin Metabiologics	415	1245×10^3	2.10^7	62	25
Complex Metabiologics	87	260×10^3	8.10^6	32.5	12
BoNT/A1 (Hall)	27.5	83	8.10^3	10.4	15
BoNT/A2 (9336)	1210	3630	10^5	36.3	19
BoNT/A3 supernatant 1 (Loch Maree)	2118	6354	10^5	64.4	16
BoNT/A3 supernatant 2 (Loch Maree)	224	672	10^4		
BoNT/A5 (126.07)	282	845	10^4	84.5	15
BoNT/A7 (148.08)	25	75	2.10^3	37.5	25
BoNT/A8 (217.12)	32	96	8.10^3	12	10

In conclusion, a new method of quantification of BoNTs in complex matrices by mass spectrometry, preferably liquid chromatography-triple quadrupole mass spectrometry-multi-

stage/mass spectrometry (LC-QqQ-MS/MS), was developed and allowed the quantification of different subtypes of BoNT/A. This quantification was used to determine, for each subtype, the lethal dose 100. The method was initially developed for the quantification of BoNT of subtype A1 but can be also applicable to subtypes A2, A3, A5, A6, A7 and A8.

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It will be apparent to those skilled in the art that various modifications and variations can be made in the practice of the present invention [and in construction of this device] without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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CLAIMS

1. A method for determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:

5 contacting a sample comprising or suspected of comprising BoNT/A with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, to isolate BoNT/A light chains;

digesting the isolated light chains with a proteolytic enzyme to create proteolytic fragments of the light chains; and

10 subjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments.

2. The method of claim 1, comprising detecting by mass spectrometry a peptide consisting of the amino acid sequence of any one of SEQ ID NOs 2 and 10-42.

15 3. The method of claim 1 or 2, wherein the method comprises:

reducing proteins in the sample;

20 contacting the sample comprising or suspected of comprising BoNT/A with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, wherein the antibody is coupled to a solid support, to form a solid support-antibody-light chain complex;

separating the solid support-antibody-light chain complex from other substances present in the sample;

digesting the light chains with trypsin to create trypsin fragments of the light chains;

separating solid support and antibody from the trypsin fragments; and

25 subjecting the trypsin fragments to mass spectrometry, in particular liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS) to determine the sequences of the fragments.

4. A method for determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:

30

contacting a sample comprising or suspected of comprising BoNT/A with antibodies specific for conserved heavy chain sequences and/or antibodies specific for conserved light chain sequences among BoNT/A subtypes, to isolate BoNT/A or the BoNT/A heavy and light chains;

5 digesting the isolated BoNT/A or the isolated heavy and light chains with a proteolytic enzyme to create proteolytic fragments of the heavy and light chains; and

subjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments.

10 5. The method of claim 4, comprising detecting by mass spectrometry a peptide consisting of the amino acid sequence of any one of SEQ ID Nos 2, 10-42 and 44.

6. The method of claim 4 or 5, wherein the method comprises:

15 contacting the sample comprising or suspected of comprising BoNT/A with antibodies specific for conserved heavy and/or antibodies specific for conserved light chain sequences among BoNT/A subtypes, wherein the antibodies specific for conserved heavy and/or the antibodies specific for conserved light chain sequences are coupled to solid supports, to form a solid support-antibody-heavy chain complex and/or a solid support-antibody-light chain complex;

20 separating the solid support-antibody-heavy and/or solid support-antibody-light chain complexes from other substances present in the sample;

digesting the heavy and light chains with trypsin to create trypsin fragments of the heavy and light chains;

separating solid support and antibodies from the trypsin fragments; and

25 subjecting the trypsin fragments to mass spectrometry, in particular liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS) to determine the sequences of the fragments.

30 7. The method of any one of claims 1 to 6, wherein the mass spectrometry is liquid chromatography-triple quadrupole mass spectrometry-multi-stage/mass spectrometry (LC-QqQ-MS/MS).

8. The method of any one of claims 1 to 7, further comprising:

comparing the sequence of the proteolytic fragments to known sequences of BoNT/A subtype sequences to determine the subtype present in the sample.

9. The method of any one of claims 1 to 8, wherein the antibody binds to a peptide selected from the group consisting of the amino acid sequences QVPVSYDSTYLSTDNEK (SEQ ID NO:3), DTFTNPEEGDLNPPPEAK (SEQ ID NO:4) and GPLGSSTDIPFQLSKYVDNQRLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN CMENNSGWKVS LNYGEIHWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNN SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL YDNQSN SGILKDFWGDY LQYDKPYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47).

10. The method of any one of claims 1 to 9, wherein the method is a diagnostic method to determine what BoNT/A subtype is present in the sample.

11. The method of any one of claims 1 to 10, wherein the sample does not include viable neurotoxin-producing bacteria or DNA from the *C. botulinum* subtype.

12. The method of any one of claims 1 to 11, wherein the sample comprises a foodstuff sample, water, a sample from a manufactured cosmetic or pharmaceutical product, an environmental, or biological sample.

13. The method for quantitatively determining a *Clostridium botulinum* neurotoxin type A (BoNT/A) subtype in a sample, said method comprising adding an internal isotypically stable standard to the sample to be analyzed, performing the steps of any one of claims 1 to 12 and quantitating the BoNT/A subtype.

14. An antibody that specifically binds to a peptide consisting of the amino acid sequence QVPVSYDSTYLSTDNEK (SEQ ID NO:3) or DTFTNPEEGDLNPPPEAK (SEQ ID

NO:4)

or

GPLGSSTDIPFQLSKYVDNQRLLSFTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK
VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
CMENNSGWKVSLSNYGEIHWTLQDTQEIKQRVVFVKYSQMINISDYINRWIFVTITNNRLNN
5 SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL
YDNQSN SGILKDFWGDYLYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV
MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL
VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47), wherein the
10 antibody is bound to a solid support.

15. The antibody of claim 14, wherein the antibody is a polyclonal antibody.

16. The antibody of claim 14, wherein the antibody is a monoclonal antibody.

17. The antibody of any one of claims 14 to 16, wherein the antibody is a rabbit
antibody.

18. A kit for determining a *Clostridium botulinum* neurotoxin type A (BoNT/A)
20 subtype in a sample, the kit comprising an antibody that specifically binds to a peptide selected
from the group consisting of the amino acid sequences QVPVSYDSTYLDNEK (SEQ ID
NO:3), DTFTNPEEGDLNPPPEAK (SEQ ID NO:4) and
GPLGSSTDIPFQLSKYVDNQRLLSFTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSK
VNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
25 CMENNSGWKVSLSNYGEIHWTLQDTQEIKQRVVFVKYSQMINISDYINRWIFVTITNNRLNN
SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDL
YDNQSN SGILKDFWGDYLYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSV
MTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNAKL
30 VASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL (SEQ ID NO:47).

19. The kit of claim 18, further comprising a solid support for binding to the antibody of the kit.

20. The kit of claim 19, wherein the solid support is a bead.

5

21. The kit of claim 20, wherein the bead is a magnetic bead.

22. Use of a method according to any one of claims 1 to 11, to determine the presence or the quantity of BoNT/A or of a particular subtype thereof present as active ingredient in a cosmetic or a pharmaceutical product.

10

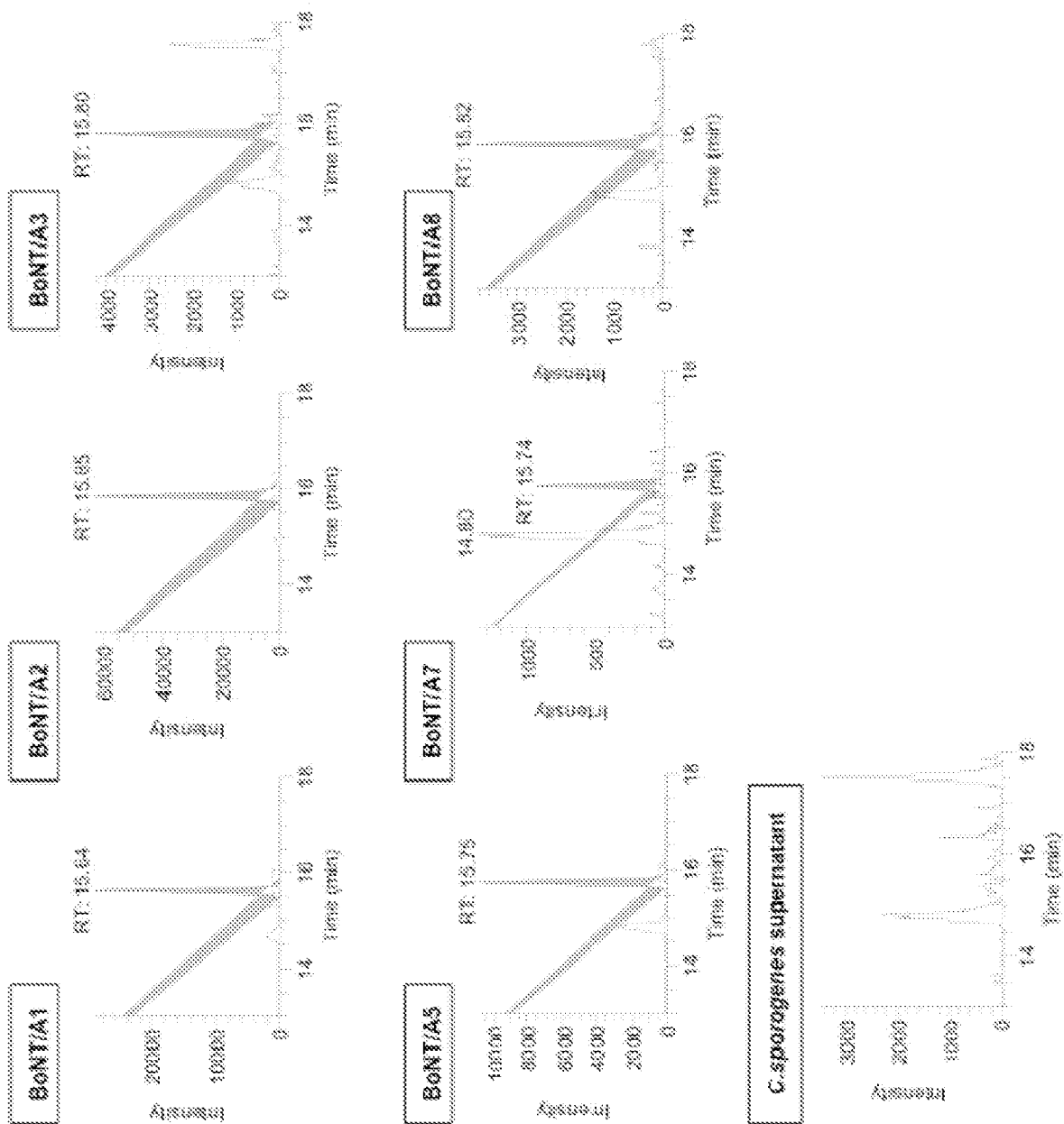


Figure 1

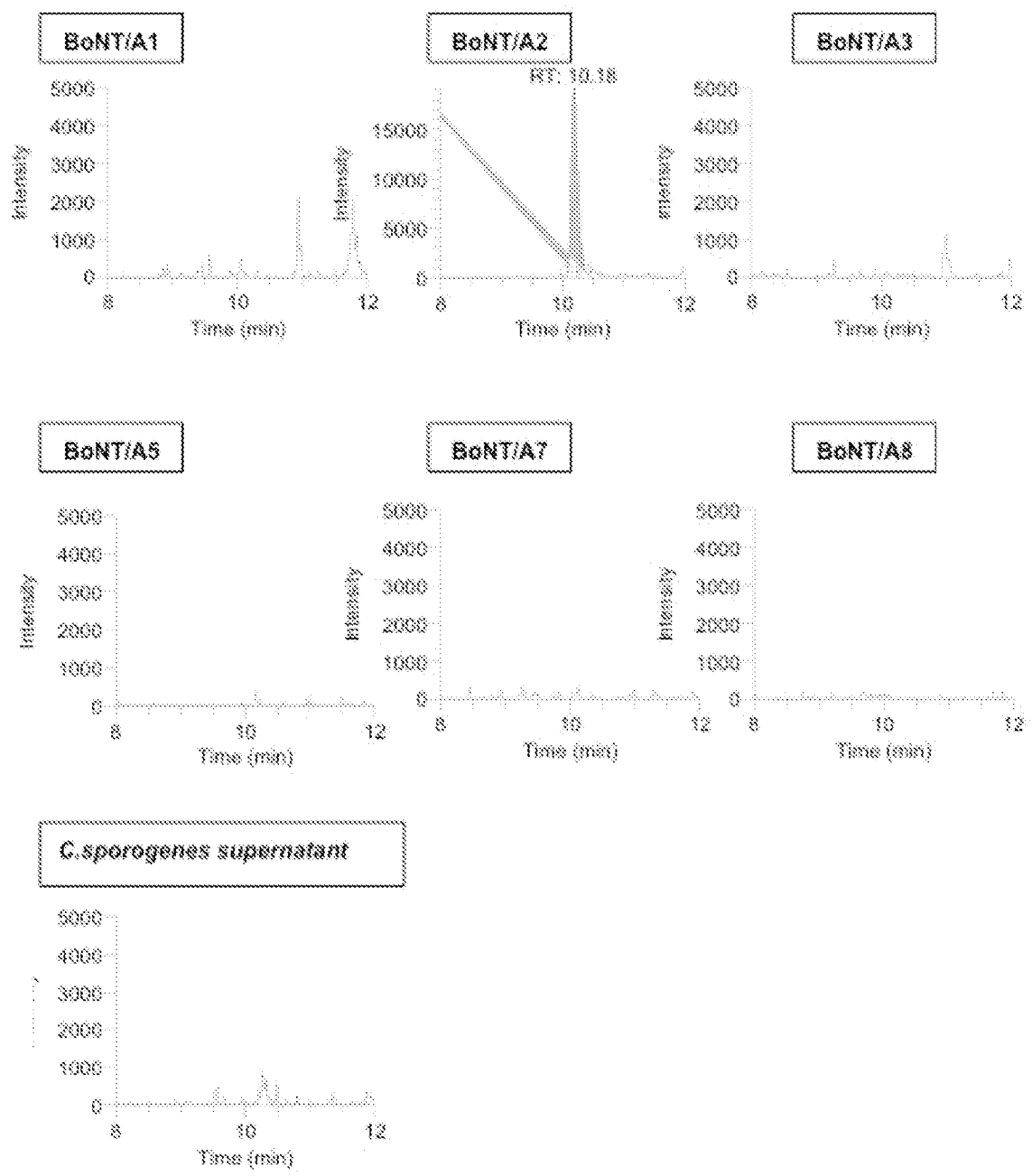
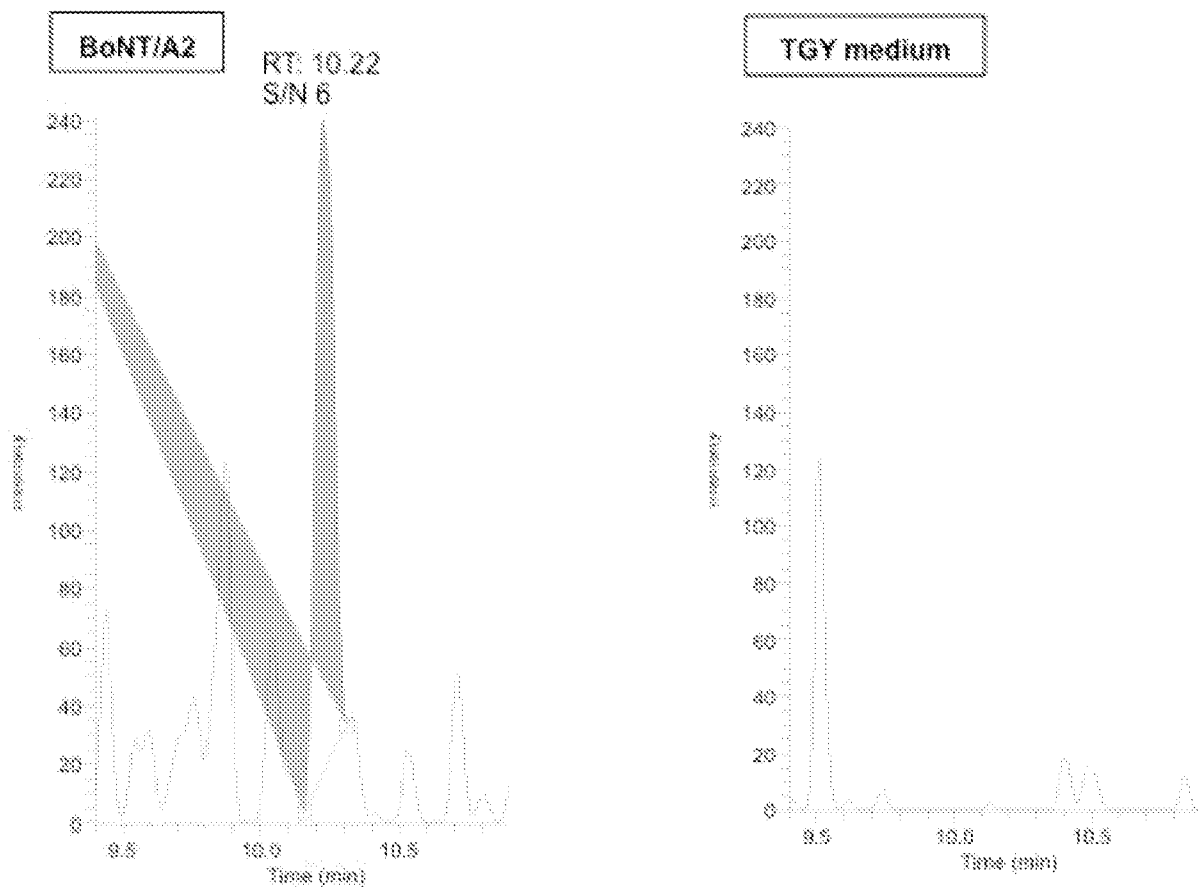
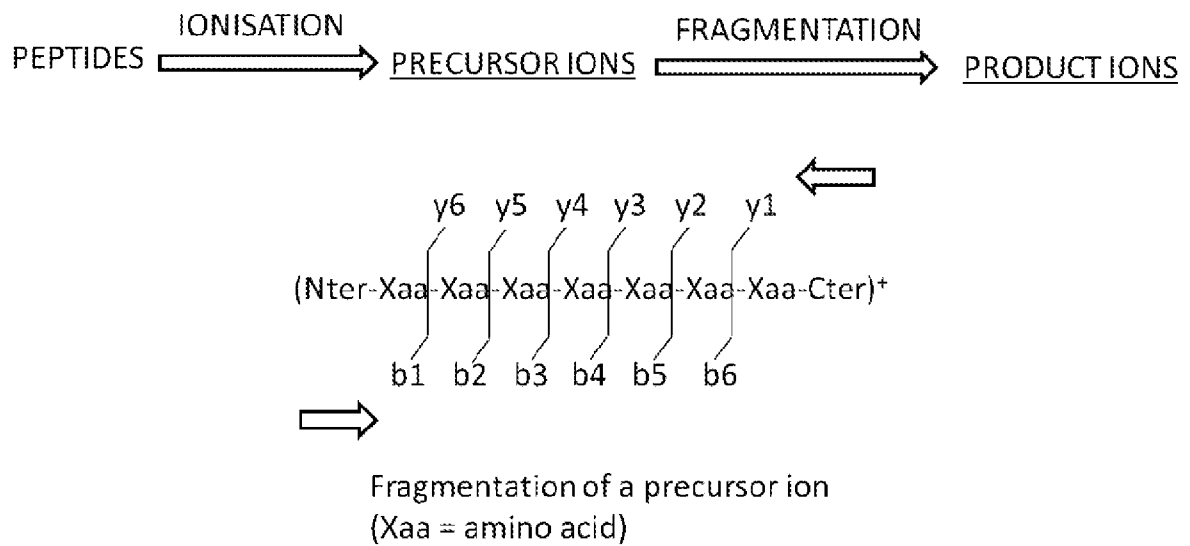
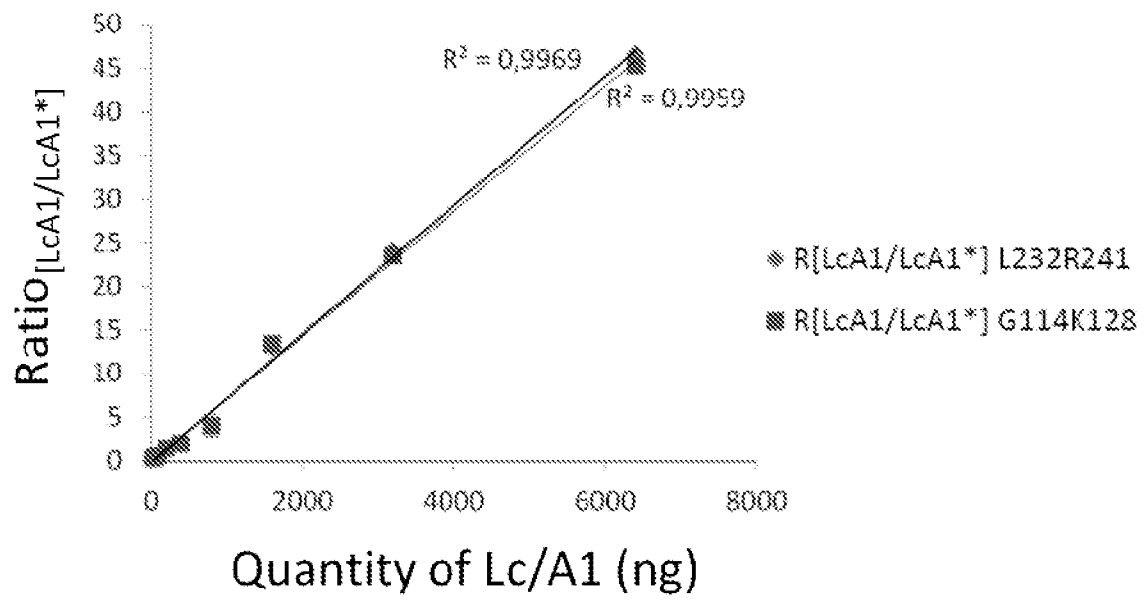


Figure 2

**Figure 3****Figure 4**

**Figure 5**

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/000784

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/569 C07K16/12 G01N33/68 G01N33/573
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZDANOVSKY ALEXEY ET AL: "Epitope mapping of botulinum neurotoxins light chains", TOXICON, vol. 60, no. 7, 1 December 2012 (2012-12-01), pages 1277-1286, XP028947201, ISSN: 0041-0101, DOI: 10.1016/J.TOXICON.2012.08.002	14,15,17
Y	figure 3 ----- -/--	16,18-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 July 2016

Date of mailing of the international search report

07/10/2016

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/000784

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BEHZOD Z. DOLIMBEK ET AL: "Regions of recognition by blocking antibodies on the light chain of botulinum neurotoxin A", IMMUNOBIOLOGY, vol. 216, no. 6, 1 June 2011 (2011-06-01), pages 698-706, XP055291131, DE ISSN: 0171-2985, DOI: 10.1016/j.imbio.2010.10.010	14,15
Y	tables 1-4	16-21
Y	----- SKADI KULL ET AL: "Multiplex Detection of Microbial and Plant Toxins by Immunoaffinity Enrichment and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry", ANALYTICAL CHEMISTRY, vol. 82, no. 7, 1 April 2010 (2010-04-01), pages 2916-2924, XP055290986, ISSN: 0003-2700, DOI: 10.1021/ac902909r	16-21
A	page 2918, column 1, paragraph 1 table 1 figure 1 page 2919, column 2, line 6, paragraph 1 - line 9 figure 2 table 2 page 2922, column 2, paragraph 3 - page 2923, column 2, paragraph 1 page 2924, column 1, paragraph 1 - paragraph 2; figure 4	1-3,7,8
A	----- SUZANNE R. KALB ET AL: "Detection of Botulinum Neurotoxin A in a Spiked Milk Sample with Subtype Identification through Toxin Proteomics", ANALYTICAL CHEMISTRY, vol. 77, no. 19, 1 October 2005 (2005-10-01), pages 6140-6146, XP055289405, ISSN: 0003-2700, DOI: 10.1021/ac0511748 tables 1-3 -----	1-3,7-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2016/000784

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-3(completely); 7-22(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3(completely); 7-22(partially)

A method for determining a Clostridium botulinum neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:contacting a sample comprising or suspected of comprising BoNT/A with an antibody specific for a conserved light chain sequence among BoNT/A subtypes, to isolate BoNT/A light chains;digesting the isolated light chains with a proteolytic enzyme to create proteolytic fragments of the light chains; andsubjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments. Antibodies and kits for use in the above methods

2. claims: 4-6(completely); 7-22(partially)

A method for determining a Clostridium botulinum neurotoxin type A (BoNT/A) subtype in a sample, said method comprising:contacting a sample comprising or suspected of comprising BoNT/A with antibodies specific for conserved heavy chain sequences among BoNT/A subtypes, to isolate BoNT/A or the BoNT/A heavy and light chains;digesting the isolated BoNT/A or the isolated heavy and light chains with a proteolytic enzyme to create proteolytic fragments of the heavy and light chains; andsubjecting the proteolytic fragments to mass spectrometry to determine the sequences of the fragments.
