Toxin Detection in Foods and Pharmaceutical Products

Food Toxin Programme: WHO case studies in mushroom poisoning

Recent development for authenticating Botulinum Neurotoxin in pharmaceutical products

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Foodborne Toxin Analytical Service

Regular Services:

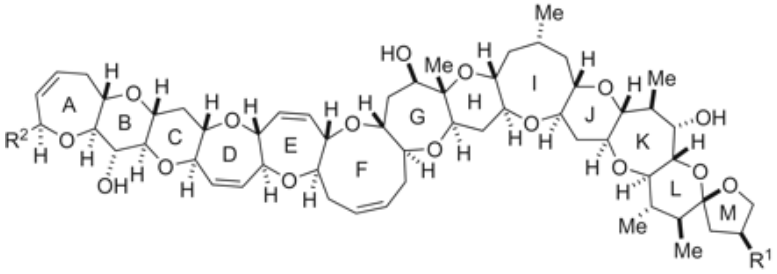
Mycotoxins
Histamine
Marine toxins
Mushroom toxins
Puffer fish toxins
Potato toxins
Natural toxins in herbs (Chinese medicine)

- To investigate & prevent food poisoning
- To protect public health for local community

Food Poisoning

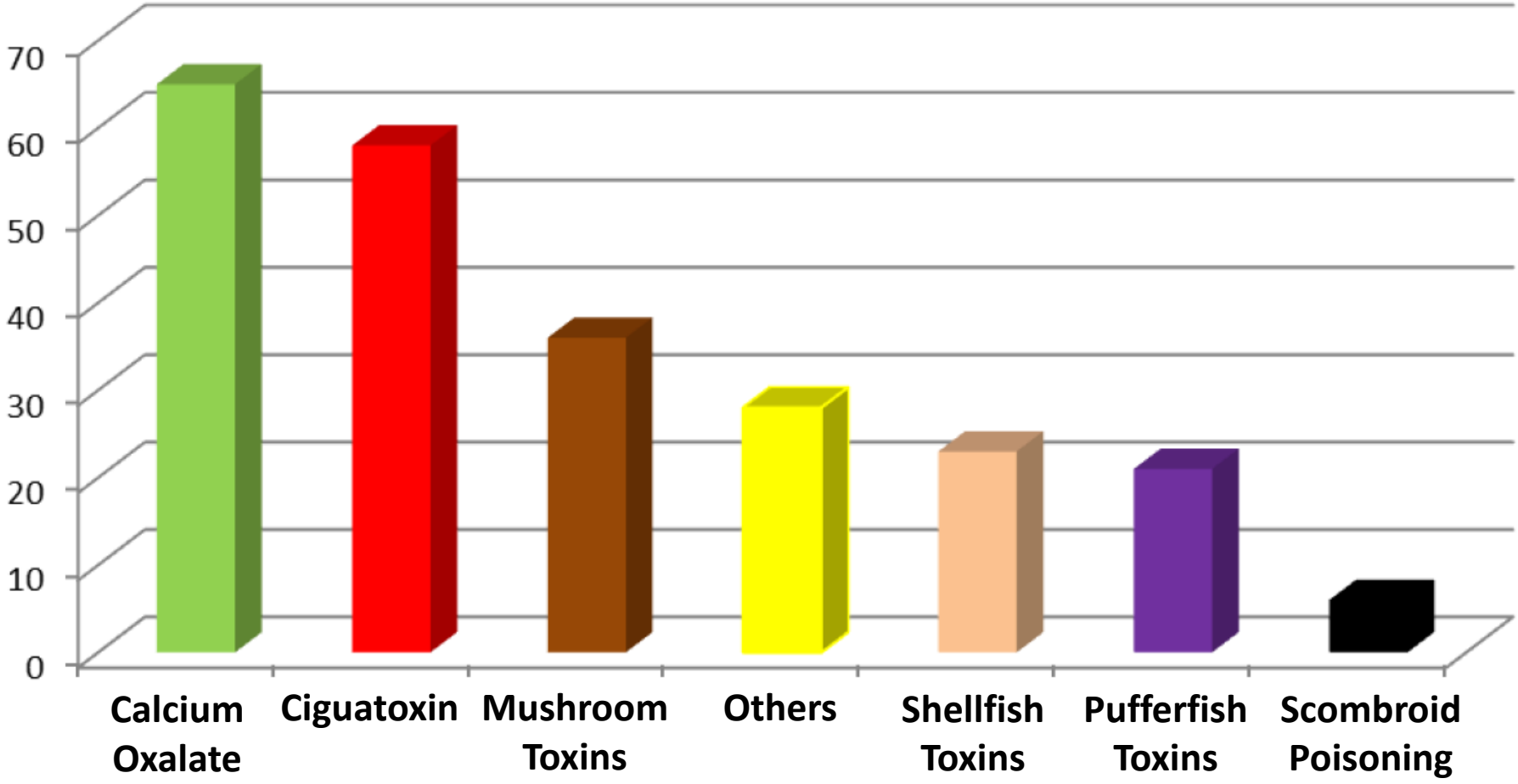
Non-chemicals: bacteria, fungus, virus, parasites, etc.

Chemicals: toxins, pesticides, heavy metals, etc.



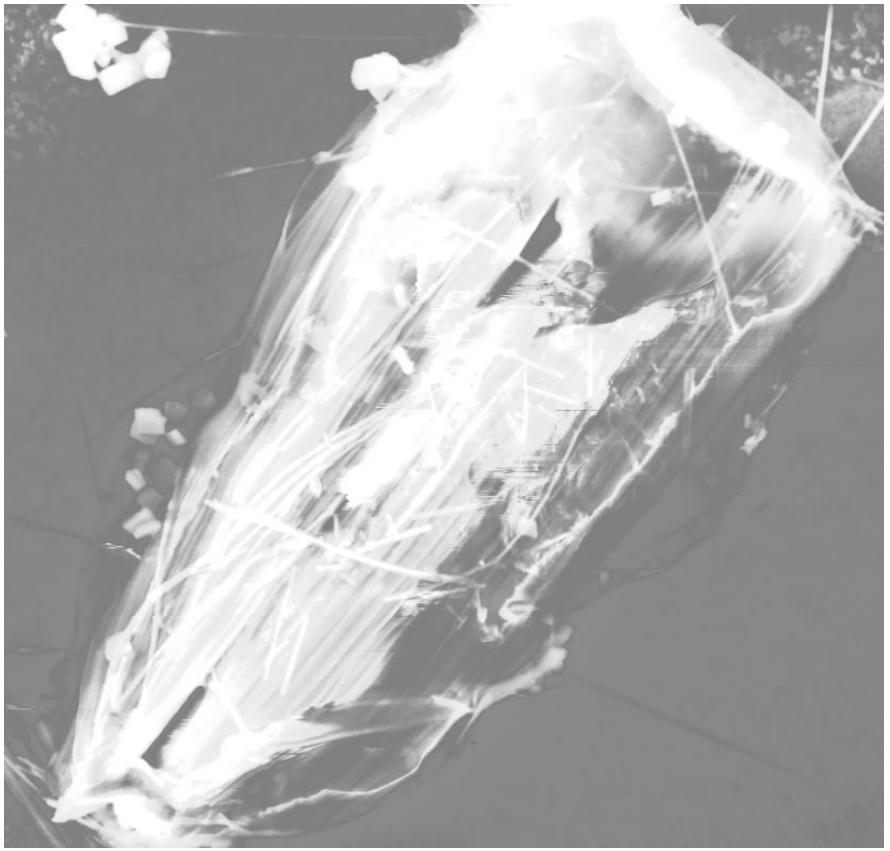
Foodborne Toxin Cases (2008 – 2013)

273 confirmed cases in Hong Kong, constituted ~8% of the total food poisoning cases



Calcium Oxalate Poisoning

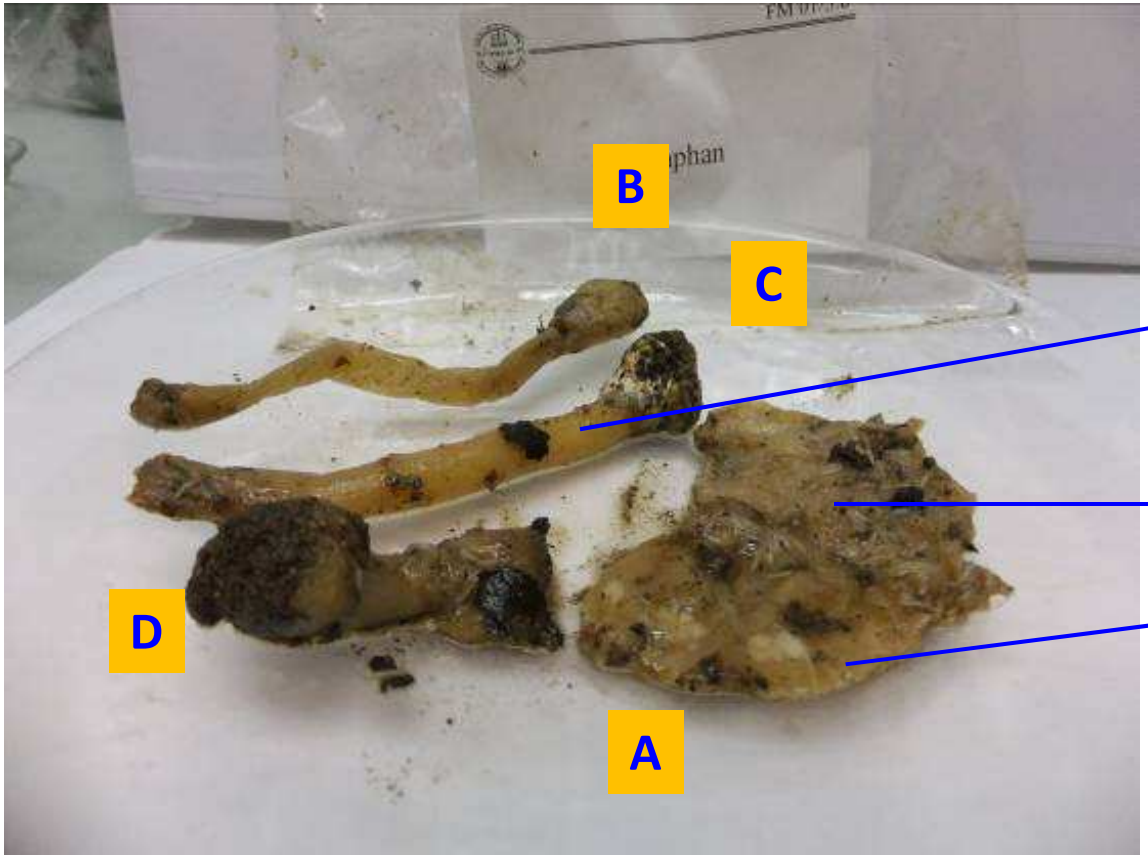
Ca oxalate needles (raphides) found in edible vegetables, water spinach, water cress
Sharp needles cause pain, swelling in oral mucosa, burning sensation



Mycetismus: WHO Cases

- Two reported fatal food poisoning cases in remote villages in the Indochina region
- Suspected eating wild poisonous mushrooms
- Local health authority claimed no capacity to test samples
- Western Pacific Regional Office contacted us for technical assistance
- Sending suspected remains of food for examination and analysis

Suspected Poisons (I)



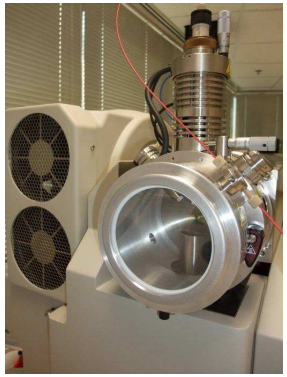
Not intact, only 4 dark yellow fragments

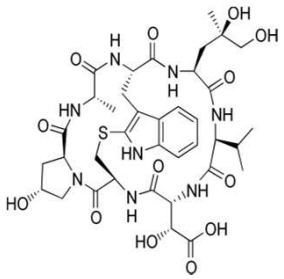
Reported symptoms: dizziness; severe vomiting; diarrhea, abdominal pain & exhausted

Involved 13 victims, 4 died (5 to 10 days)

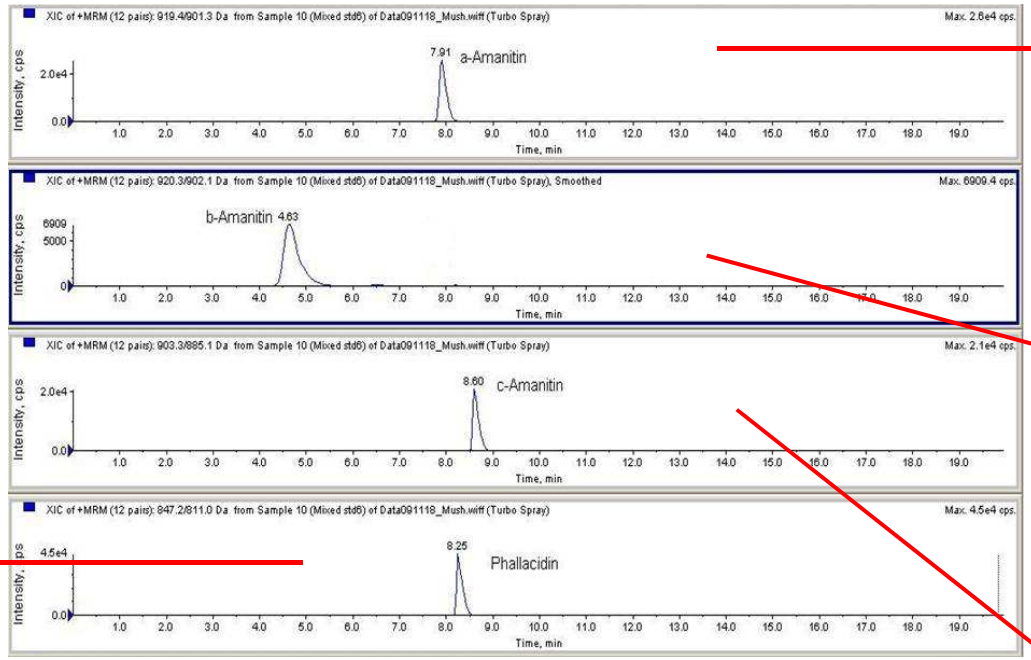
LC-MS/MS Analysis

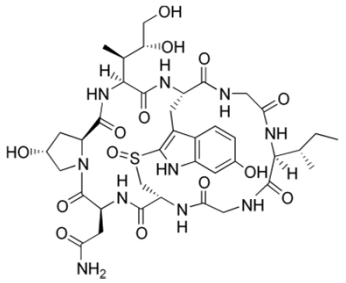
An accreditated method for detecting common toxins: amanitins, phallacidins and muscarine



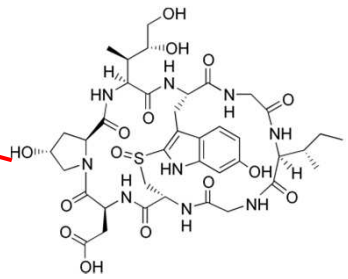


Phallacidin

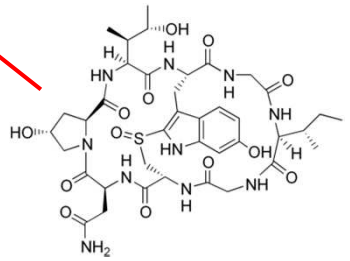




α-Amanitin



β-Amanitin



γ-Amanitin

Detection limit: 0.25 mg/kg

Mushroom Toxin (mg/kg)

Toxins	Parent Ion	MRM	A	B	C	D
α-Amanitin	919.4	901.4*, 259	560	370	350	360
β-Amanitin	920.4	902.5*, 259	190	140	120	100
γ-Amanitin	903.4	885.3*, 243	0.9	1.6	0.9	0.8
Phallacidin	847.3	811.3*, 829	150	110	110	150
Phalloidin	789.3	753.3*, 771	ND	ND	ND	ND
Muscarine	174.1	115.1*, 97	ND	ND	ND	ND

Very high level of the most deadly α-amanitin was detected

Lethal dose: ~10 mg for average-sized adults

Presence in some toxic species of ***Amanita*** genus

Toxic Culprit

DNA analysis showed that it is ***Amanita exitialis***

TGCGGAAGGATCATTAATGAAATGAATCTTGAGGCTGTC
GCTGGCCCATCTGGGCATGTGCACGTCTCTGGTCATTACC
AATTCCACCTGTGCACACTTGTAGACACTTGGGAATGAGA
GGCTTTGACCAGTCTCTTGAGAAGTTGAAATCTGGGTGTC
TATGGCATTTTATTAAACACTAGTTGCATGTTTATAGAATG
ATGATTTGAATATATATATATATATAAAGTACAACTTTCAA
CAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGCATTCC
AAGGAGCATGCCTGTTTGAGTGTCATTAAAGTCTCAAGAC
CTGTCTGATTTTGATAGGTATTGGATTTTGGGGGTTGCAG
GCTTTTTCAGACTGCCTGCTCTCCTTGAATGTATTAGTGGA
GAAAAAGCCATTTGAACTCCATTGGTGTGATAAAATCTAT
CAATGCCAGGAGCAATGCTAGTAATCTCTGCTGTCTAACT
GTCTGTAAAAATGGACAATTTGACCAACTTGACCTCAAAT
CAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGC
GAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCAGA

Known as Guangzhou destroying angel

- Onset of action: 6 – 18 hrs
- Latent period: 2 – 4 days (false recovery)
- Intoxication stage: liver and kidney failure, black urination, yellow skin, loss of strength
- Liver transplant is necessary

Common Deadly Mushrooms

Death cap
-*A. phalloides*,
-native to Europe but found widespread

Destroying angels
- *A. verosa*

Fool's mushroom
- *A. verna*

Recent Local Fatal Cases

In 2014, 5 severe hospitalized cases for local residents:

- 3 Destroying angel
- 1 Fools' mushroom (China)
- 1 Death cap (S. Africa)

  1 dead, 1 required liver transplant

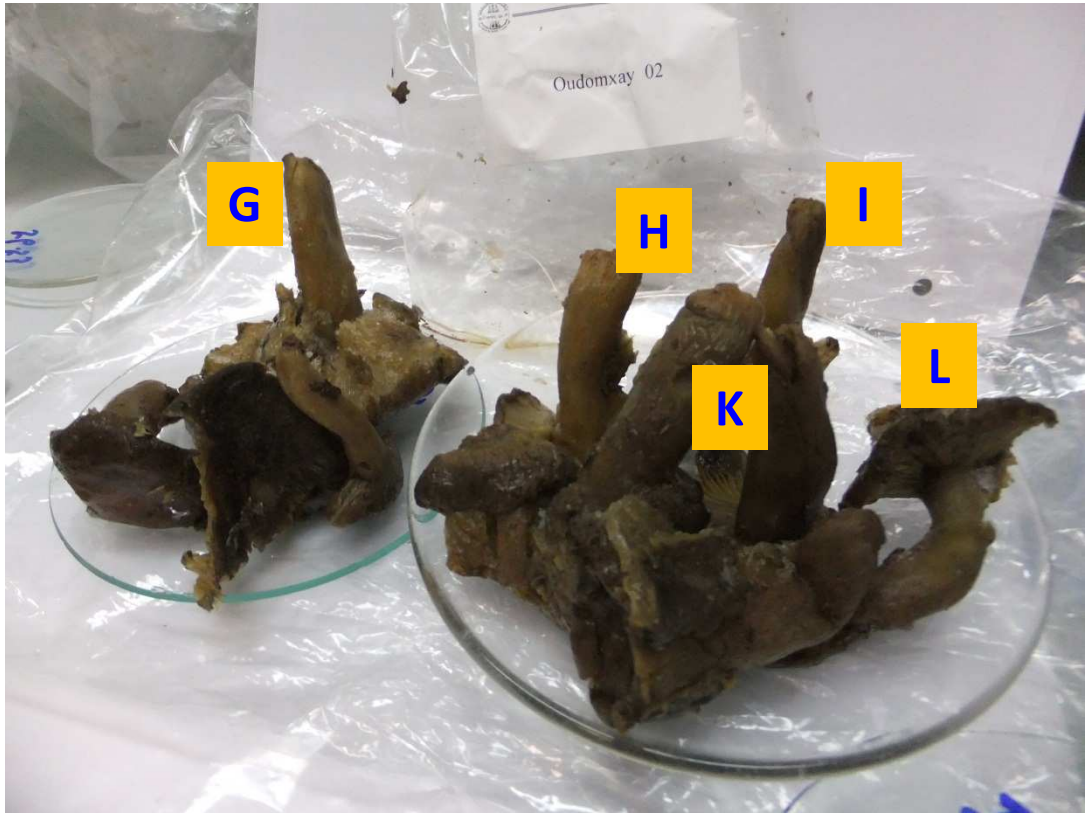
Erroneous Folklore

(from Wikipedia)

- Poisonous mushrooms are brightly colored

- Poisonous mushrooms have a pointed cap, edible ones have a flat, rounded cap
- Poisonous mushrooms blacken silver
- Poisonous mushrooms taste bad
- Poisonous mushrooms will turn rice red when boiled
- Mushrooms are safe if thoroughly cooked
- ……

Suspected Poisons (II)



Quick onset: 10 min

Vomit, sore throat, headache, difficult breathing, body paralyze

8 victims, 1 died (2 days after consumption)

Analytical Results

Toxins	G	H	I	K	L
α-Amanitin	ND	ND	ND	ND	ND
β-Amanitin	ND	ND	ND	ND	ND
γ-Amanitin	ND	ND	ND	ND	ND
Phallacidin	ND	ND	ND	ND	ND
Phalloidin	ND	ND	ND	ND	ND
Muscarine	ND	ND	ND	ND	ND

DNA analysis showed it was ***Russula subnigricans***

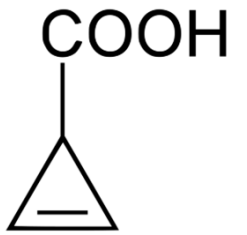
Russula Subnigricans

Literature information:
Contains trace or no amanitins and phallicidins

Cycloprop-2-ene carboxylic acid is the major component to cause toxic effects

Cycloprop-2-ene carboxylic acid

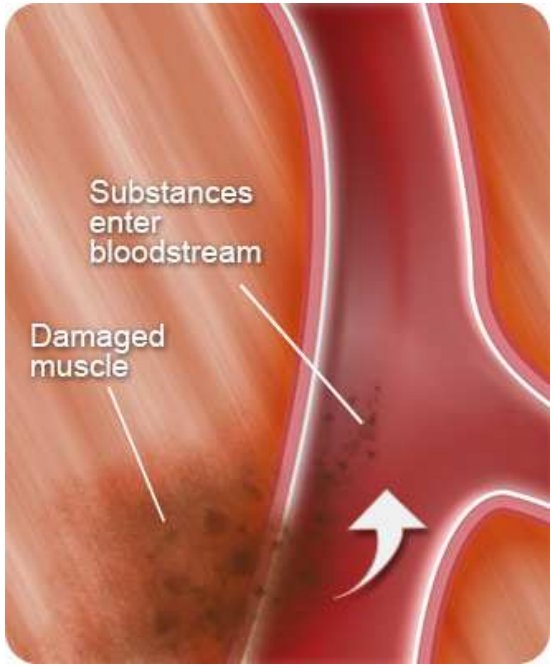
Almost Impossible to detect *in vitro* by instruments →



Nature Chem Bio 5, (2009) 465 - 467

- A highly strained 3-carbon ring natural compound
  → **Rhabdomyolysis**

- Breakdown of skeletal striated muscles
- Substances enter bloodstreams
  → tea coloured urine in 12 -14 hrs
- Kidney failure, collapsed
- Matched with WHO's descriptions



Mutually Beneficial

- Analytical report:
  Case I: *Amanita exitialis* containing α-, β-, γ-amanitin & phallacidin
  Case II: *Russula Subnigricans*

- WHO set up preventive measures, eg. educational programmes to teach villagers on wild mushrooms

- Possibility of technology transfer or technical training

- GL: Invaluable lesson to mushroom toxins

Authentication of Botulinum Toxin (BoNT or Botox) in Pharmaceuticals

Fake Botox Products

News › China › Policies & Politics

Beware of fake Botox injections, China's drugs watchdog warns

Warning comes following several people's hospitalisation after receiving dodgy beauty treatment

PUBLISHED : Friday, 03 June, 2016, 1:08pm
UPDATED : Friday, 03 June, 2016, 11:17pm

Comments:

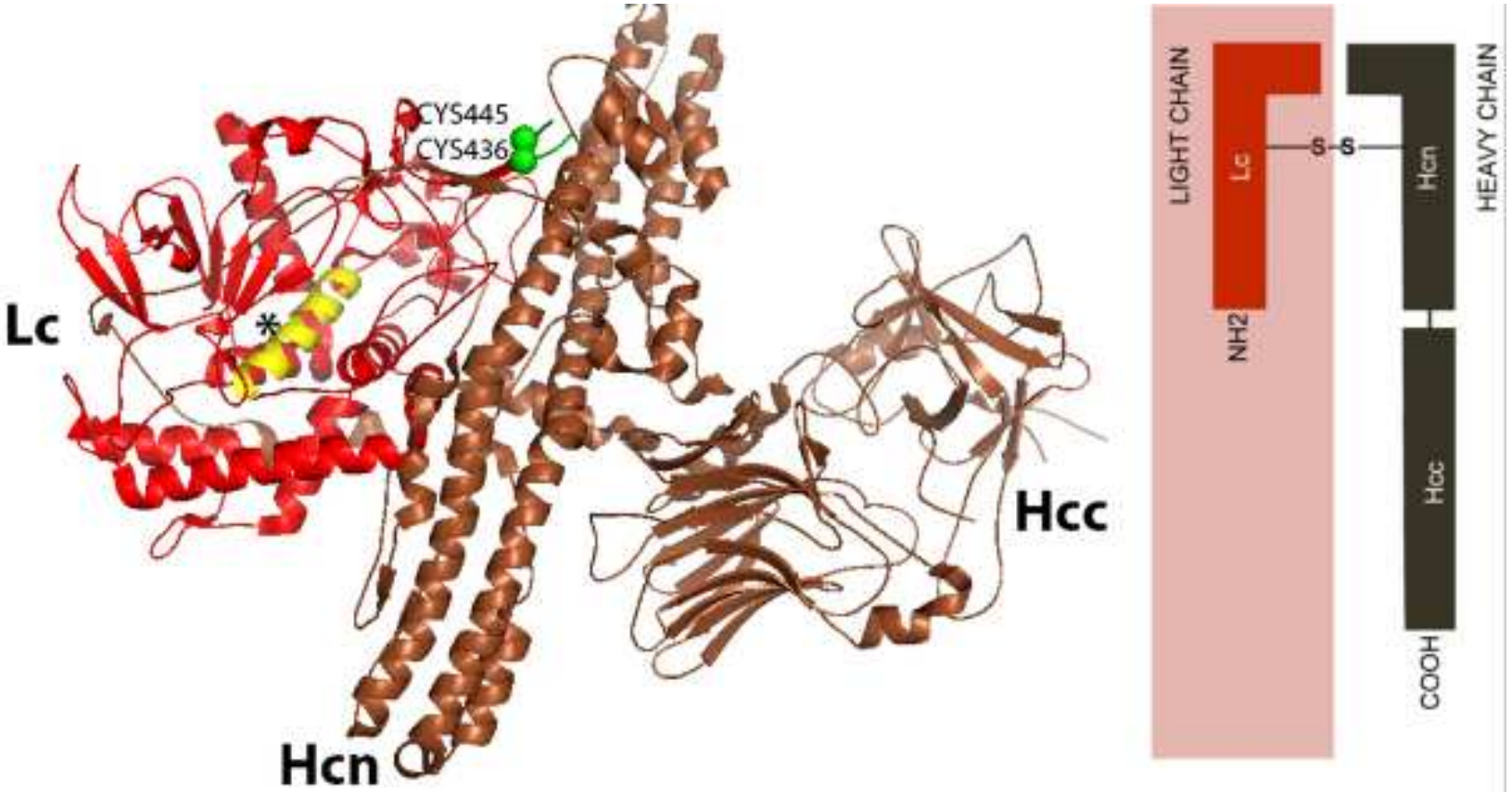
Latest news on 3 Jun 2016 about extensive use of fake botox in China

Introduction: Botox

- Produced by *Clostridium Botulinum*
- Seven botox serotypes (A-G)
- Botox causes botulism in human by inhibiting the release of a neurochemical transmitter, acetylcholine (Ach) leads to nerve blockade and muscle disorders
- Onset: 18 – 36 hrs
- Symptoms: Blurred vision, drooping eyelids, slurred speech, difficulty swallowing, muscle weakness
- Infection via wounds, contaminated food, spores

3D Structure of Botox

- A protein containing about 1,300 amino acids
- MW of Botox A : 150kDa (Heavy chain ~100kDa + a light chain ~50kDa via a disulfide bridge)

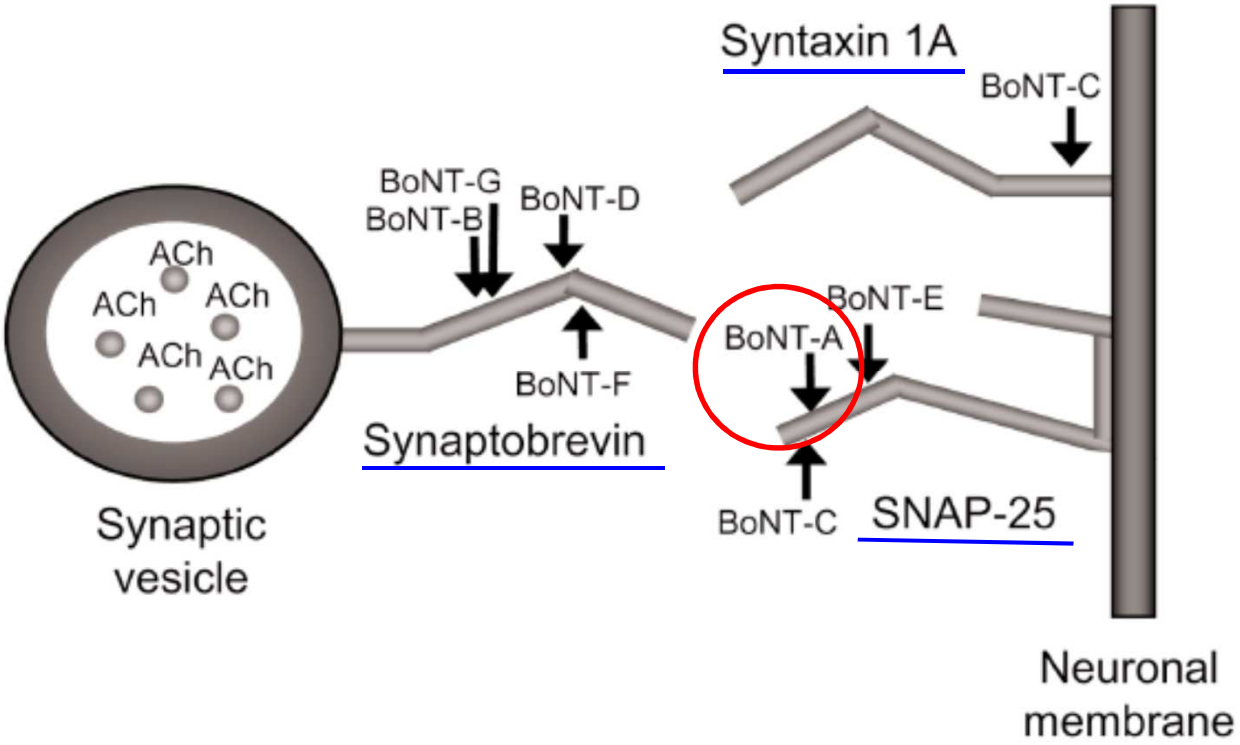


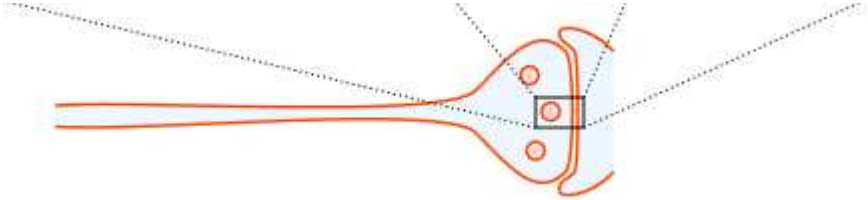
Biochemical Weapon Choice

Highly toxic: LD_{50} ~ 0.0002 mg/kg; estimated 1 g could kill 1 million people (an ideal biochemical weapon)

AGENT	LD_{50} (mg/kg)	SOURCE
Botox	0.0002	Bacterium
Tetanus Toxin	0.002	Bacterium
Ricin	3	Castor Bean
VX	15	Chemical Agent
Soman (GD)	64	Chemical Agent
Sarin	100	Chemical Agent

Action of Botox





Axon - Synapse

Emerging Infectious Diseases (2005) **11**: 1578-1583

Clinical Applications

- Diluted Botox preparations have been applied to treat different symptoms since late 1960s:

Ophthalmology: blepharospasm, strabismus

Upper motor neuron syndrome: spasticity

Excessive sweating

Chronic migraine

- Came to cosmetic industry, relieve facial wrinkles. Botox A was first used to remove glabellar frown lines in USA in 1989

Beauty Business (wonder drug)

- USFDA approved Botox A (BOTOX®) in April 2002 for removal of facial winkles
- Projected a global market at > USD 4 billions in 2018
- Also registered as pharmaceutical products in Hong Kong

Registered Products

BOTOX A PRODUCTS	COUNTRY OF ORIGIN	DOSAGE	EXCIPIENTS
Botox	USA	100 IU 200 IU	HSA (0.5 mg) NaCl (0.9 mg)
Xeomin	Germany	50 IU 100 IU	HSA (0.5 mg) Sucrose (4.7 mg)
Dyspot	UK	300 IU 500 IU	HSA (0.5 mg) Lactose (2.5 mg)
Siax	Korea	100 IU	HSA (0.5 mg) NaCl (0.9 mg)
BTXA	China	50 IU 100 IU	Gelatin (5 mg) Dextran (25 mg) Sucrose (25 mg)

By law, registered botox should be distributed to use on human by a licensed medical practitioner

Analysis of Botox

- Product (Injection formulation) compliance tests listed in pharmacopeias (eg. BP, USP) or AOAC Official Method (977.26) for consistency, stability, safety and efficacy using *in vivo* bioassay, however ….

Large number of mice

4-day monitoring for symptoms

Inhumane

Pressure from animal welfare organizations

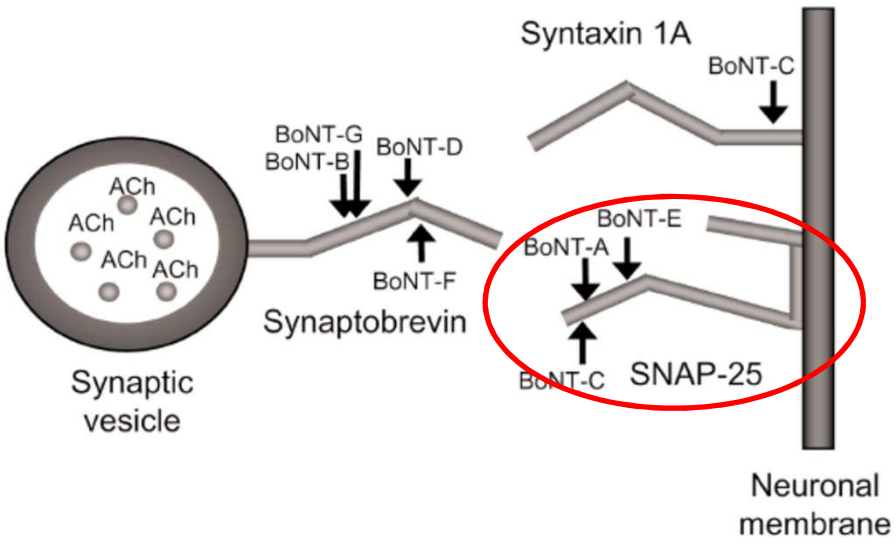
Instrument Analysis?

- Intrinsic issue: Large MW (150kDa)
- Extrinsic issue: ultra-trace amount (pg to fg); severe interference from stabilizing proteins (mg)
- Health issue: extremely toxic, specialized laboratory facility required
- Chemical methods not easy; limited literature info

  NIBSC – ELISA

  USCDC – LC-MS/MS with sophisticated reagents, US patented

Our Approach



(a) Use modified SNAP-25 protein (substrate), 187-203 (17 aa)

(b) Add to botox A in buffer medium

(c) Botox A cleaves the substrate forming two fragments

(d) Confirmation of fragments by MALDI-TOF, LC-MS/MS or LC-TOFMS

(e) Indirect detection of Botox A

Endopep-MS Method

---D-M-G-N-E^{170}-I-D-T-Q-N^{175}-R-Q-I-D-R^{180}-I-M-E-K-A^{185}

-D-S-N-K-T^{190}-R-I-D-E-A^{195}-N-Q^{197}-R^{198}-A-T^{200}-K-M-L-G-S^{205}-G

187 203

Botox A

SNKTRIDEANQ^{197}-R^{198}ATKML
(modified substrate)

17 aa

11 aa

SNKTRIDEANQ^{197}
(N-terminal product)

+

R^{198}ATKML
(C-terminal product)

6 aa

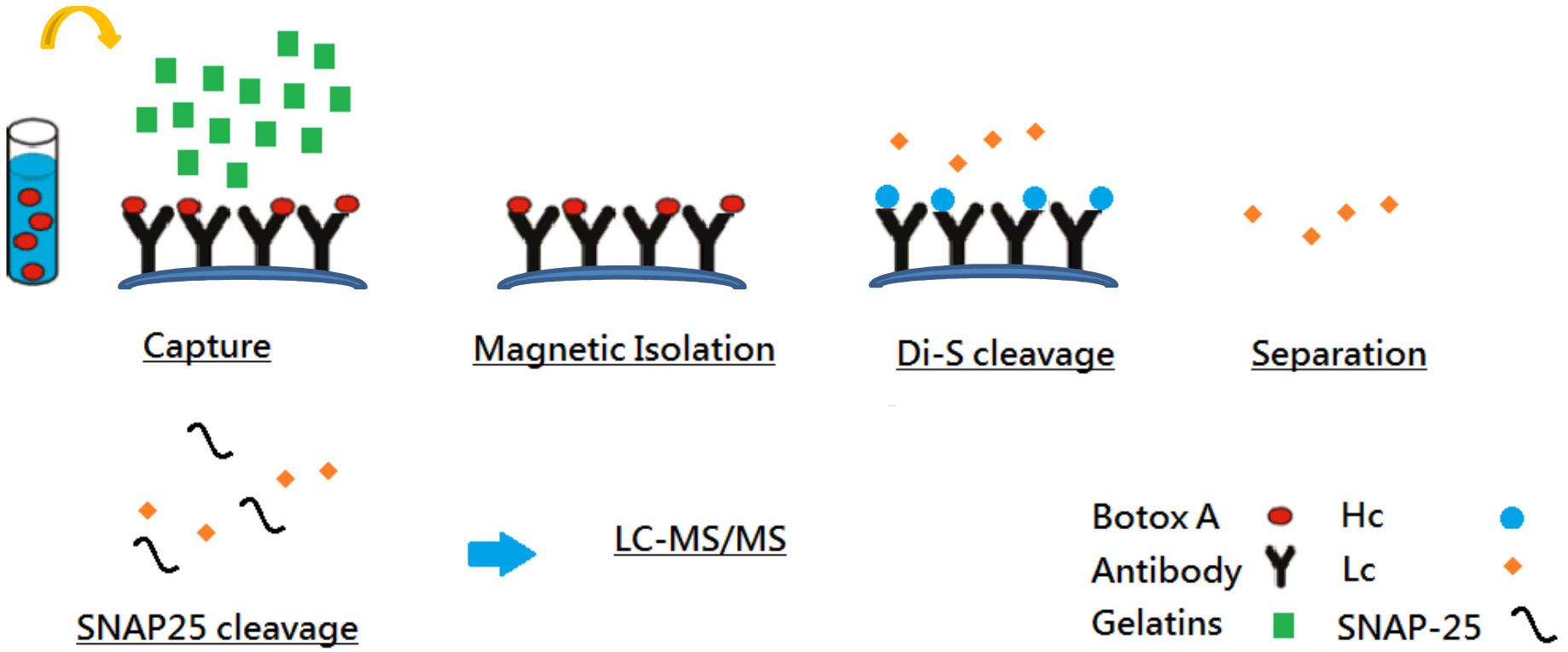
LC-MS/MS

MALDI-TOF

Designed Experiments

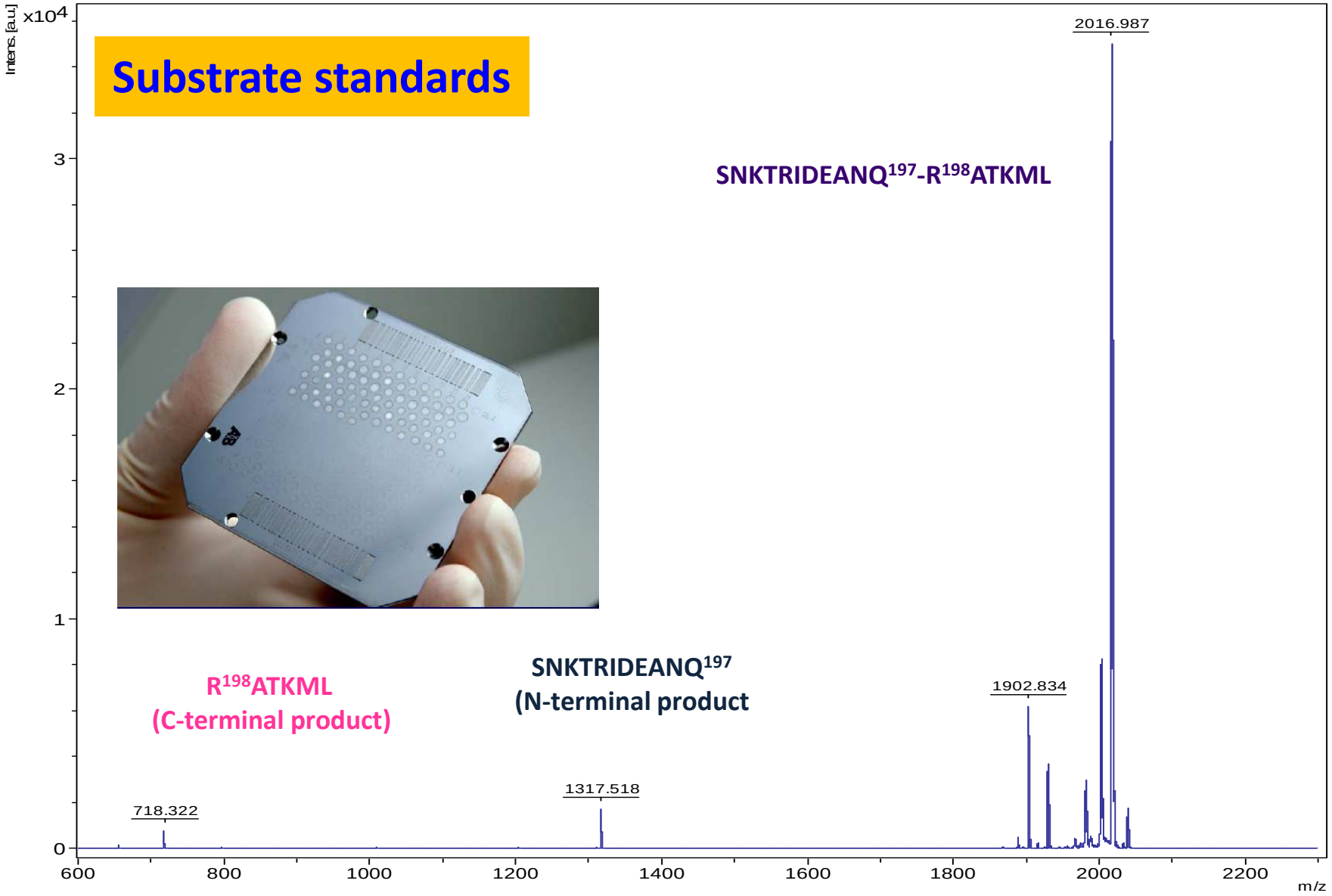
1. Capture: separation of botox A from matrix
2. Cleave disulfide bridge to free Lc of botox A
3. Optimize *in vitro* proteolytic conditions of Lc on the modified substrate: buffer, duration, temp, amount of substrate used, possible interference study, etc.
4. Optimize LC columns for good separation
5. Detection: MALDI-TOF and LC-MS/MS systems
6. Source out biotech companies to synthesize substrate and substrate fragments

Capture Technique



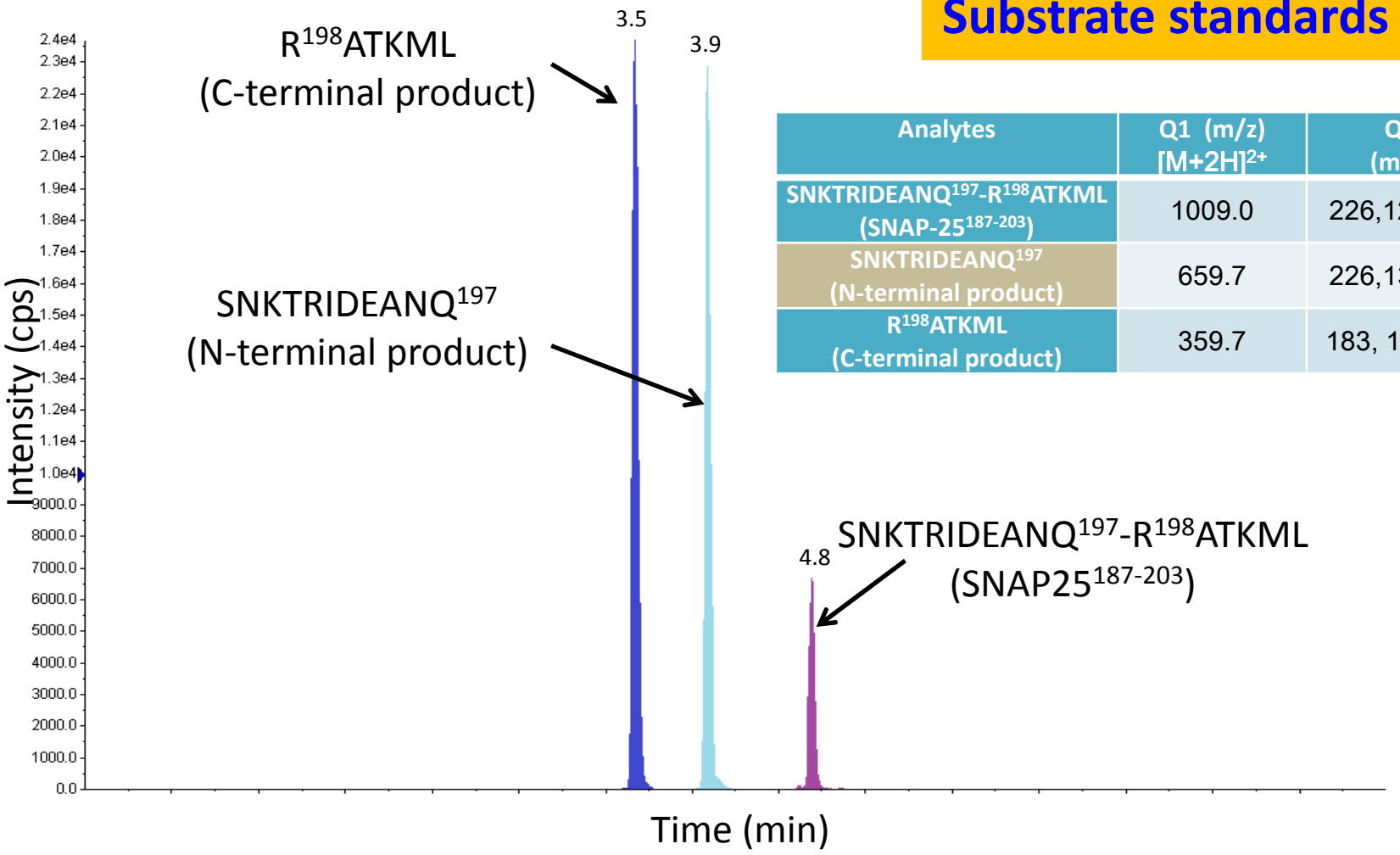
- Using polyclonal Ab coated magnetic beads
- Effective removal of matrices interference
- Improved capturing botox A

MALDI-TOF


Intens. [a.u.]
x10^4
3
2
1
0
Substrate standards
2016.987
SNKTRIDEANQ197-R198ATKML
R198ATKML
(C-terminal product)
SNKTRIDEANQ197
(N-terminal product
1902.834
1317.518
718.322
600
800
1000
1200
1400
1600
1800
2000
2200
m/z

API Q-Trap

Substrate standards



Analytes	Q1 (m/z) $[M+2H]^{2+}$	Q3 (m/z)
SNKTRIDEANQ197-R198ATKML (SNAP-25187-203)	1009.0	226,129, 84
SNKTRIDEANQ197 (N-terminal product)	659.7	226,130, 84
R198ATKML (C-terminal product)	359.7	183, 129, 84

MRM chromatogram for SNAP-25$^{187-203}$ substrate and the corresponding cleavage product standards using C18 column

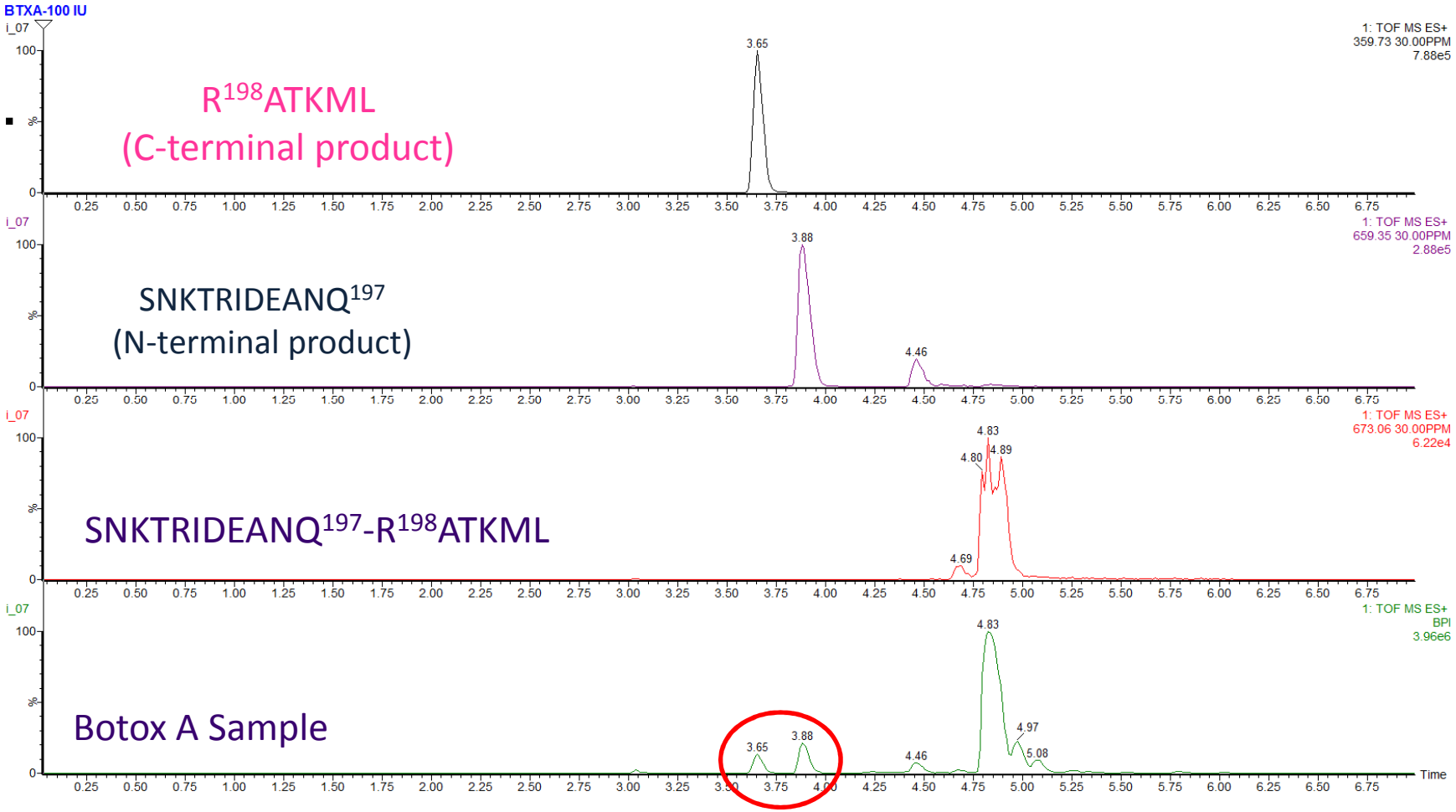
Optimization for Real Samples

Reaction Conditions	HEPES (M)	DTT (mM)	$ZnCl_2$ (mM)	BSA (mg/mL)	Substrate (nmol)
1	0.05	25	0.025	1	1
2	0.05	25	0.25	1	1
3	0.05	25	2.5	1	1
4	0.05	25	25	1	1
5	0.05	5	0.3	1	1
6	0.05	5	2.5	1	1
7	0.05	1.5	0.3	1	1
8	0.05	1.5	2.5	1	1
9	0.05	5	1	1	1
10	0.05	1.5	0.75	1	1
11	**0.05**	**5**	**2.5**	**1**	**5**

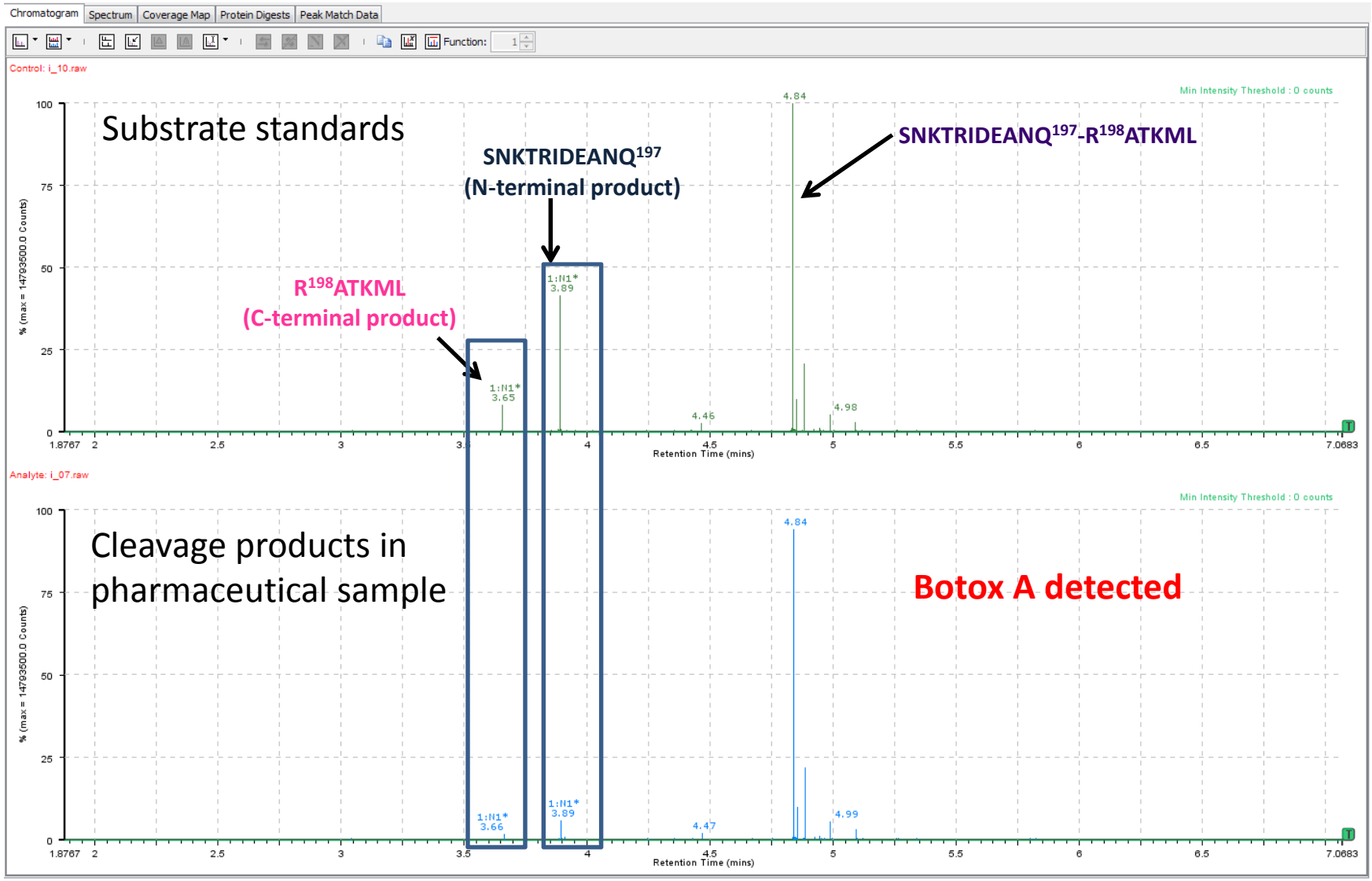
HEPES: buffer for all reactions; DTT: cleave S-S; $ZnCl_2$: cleave substrate;
BSA: major excipient
Reaction time investigation

SYNAPT G2 Q-TOFMS

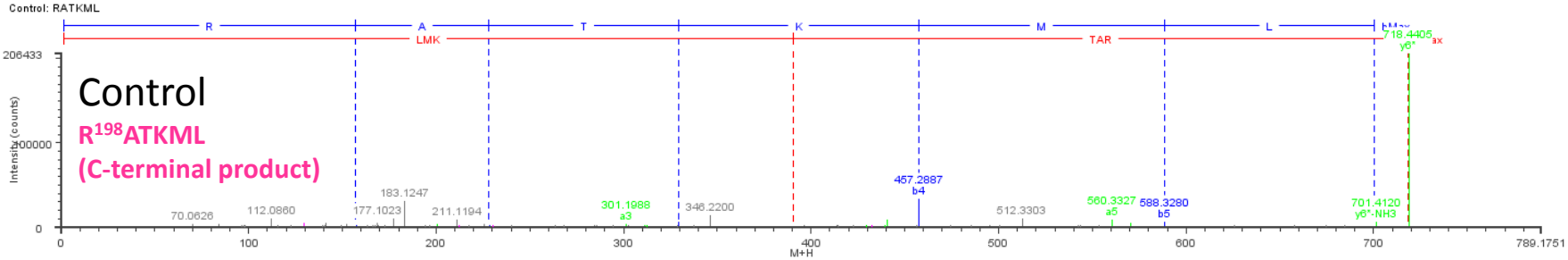
Cleavage products produced by real sample BXTA (Botox A 100 IU)

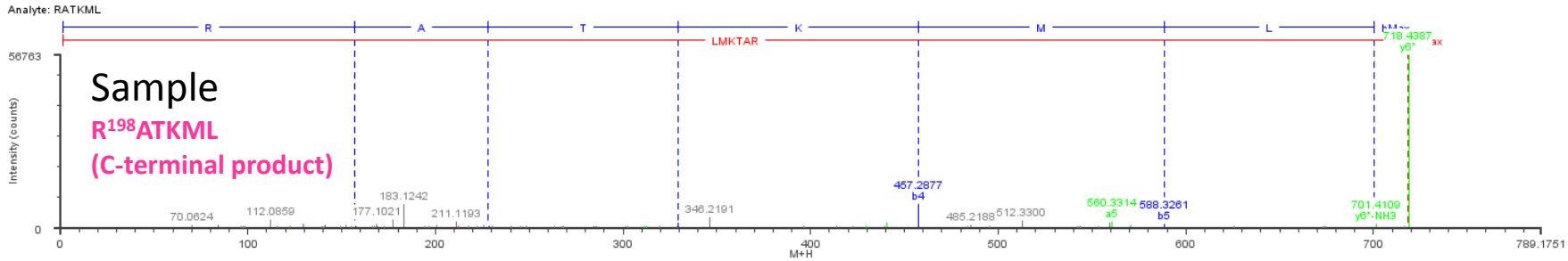


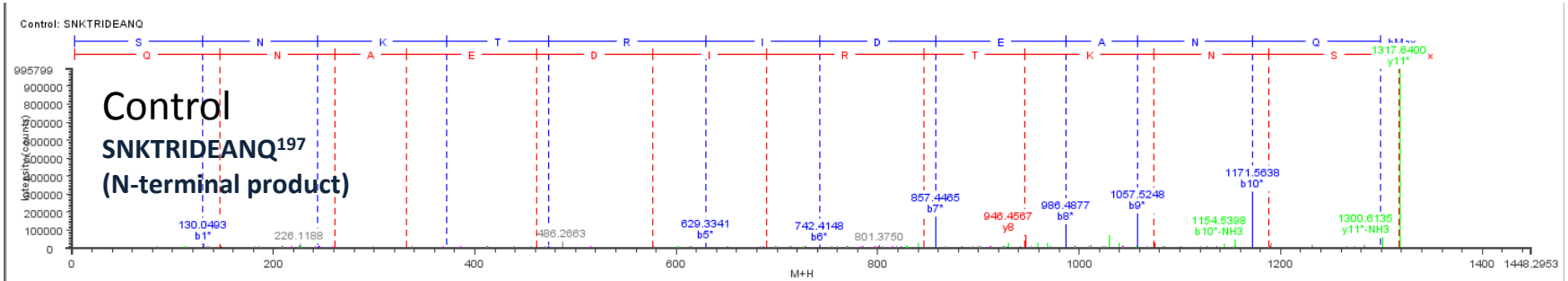
SYNAPT G2 Q-TOFMS

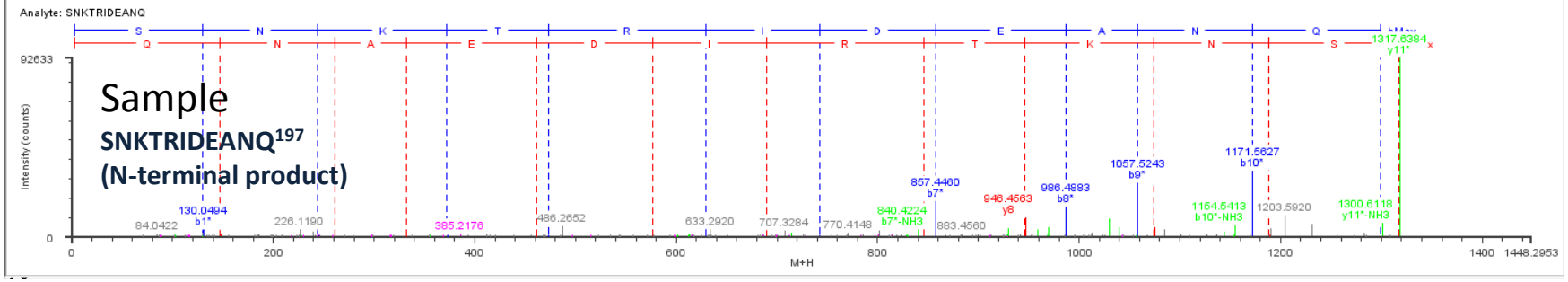


Confirmation (Q-TOF MS/MS)







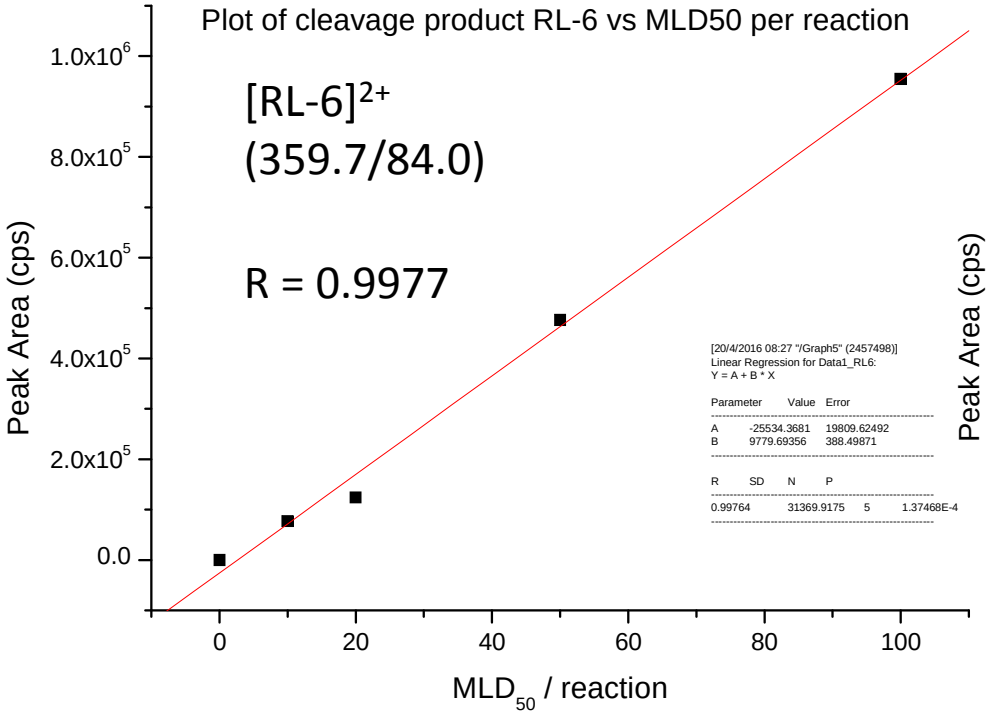


Calibration of Two Fragments

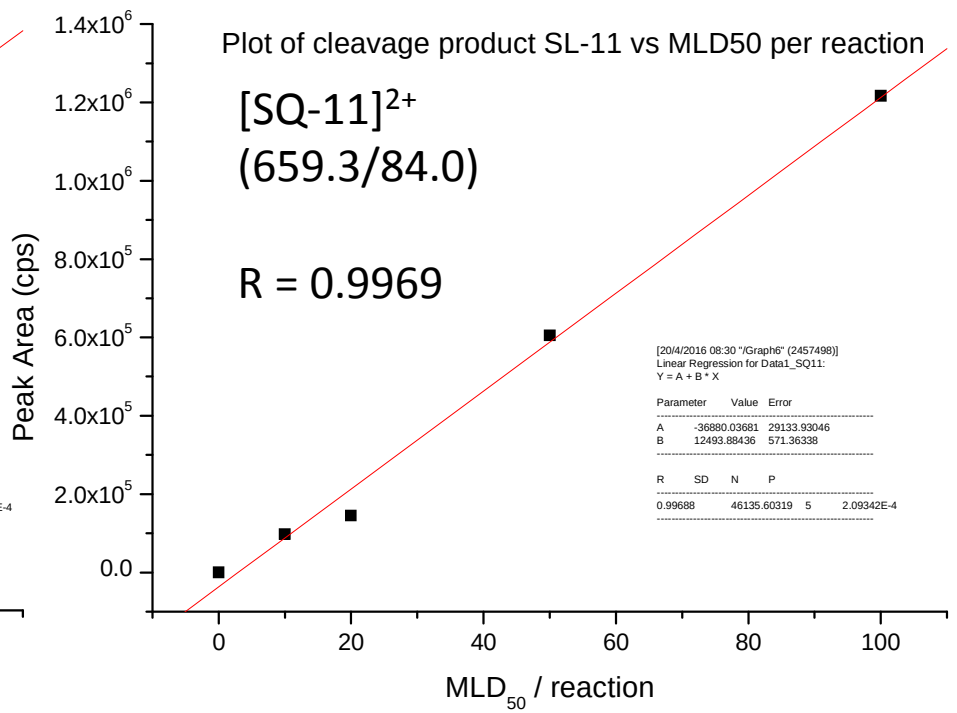
For quantitative purpose

Linear range = 10 – 100 IU

R^{198}ATKML
(C-terminal product)



SNKTRIDEANQ^{197}
(N-terminal product)



What We Achieved

1. Positive identification of Botox A in all registered pharmaceutical products
2. Linear calibration curves established for quantitation
3. Reporting limit = 10 IU
4. Analytical service provided to law enforcement authorities in autumn 2016
5. Possibly replace animal test
6. Authenticate botox A in pharmaceutical samples

Future Work

1. Extend to measure botox A and other serotypes in food matrices to investigate possible botulism

2. Develop a Direct quantitative method by looking at signature peptide marker in botox A (in progress)

THANK YOU