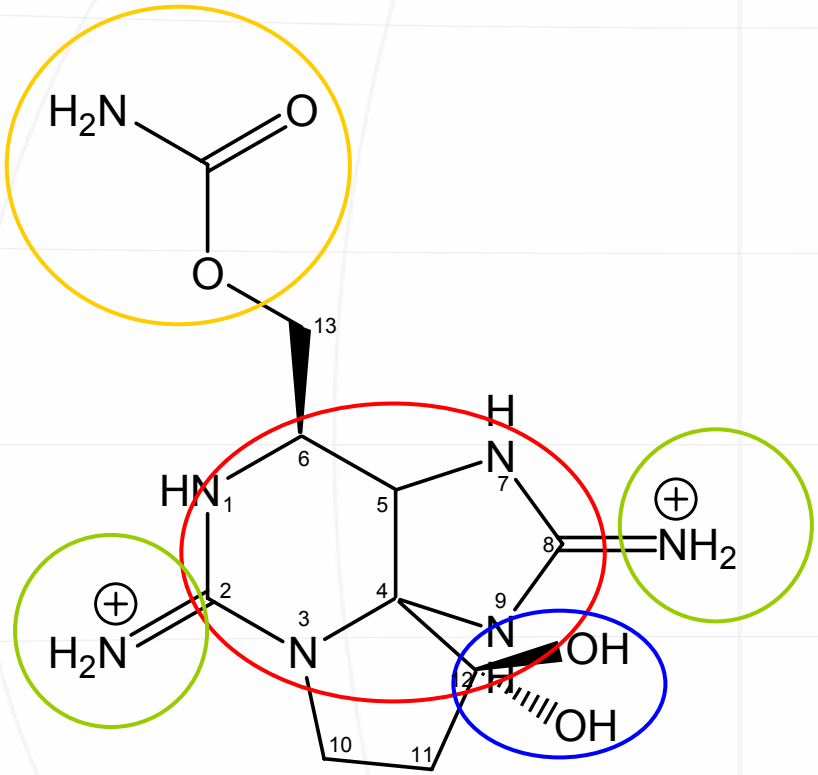
Practical Aspects of the Determination of Paralytic Shellfish Poisoning (PSP) Toxins by Liquid Chromatography-Fluorescence Detection

Outline

1. Chemistry of PSTs
   - structural characteristics
   - 11-sulfate keto-enol tautomery
   - oxidation of PSTs
   - hydrolysis of B- and C-toxins
2. Chromatography
   - post-column derivatization
   - retention
   - sensivity
3. Parameters and their impacts
   - hydrolysis: temperature, time
   - oxidation: pH
   - column: temperature
   - eluants: pH
   - injection: volume
   - matrix effects, imposters
4. Alternative methods
   - Oshima
   - Lawrence
5. Conclusions

Saxitoxin – Chemical Structure

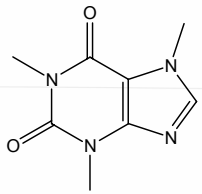


Purine derivative

2 imino functions

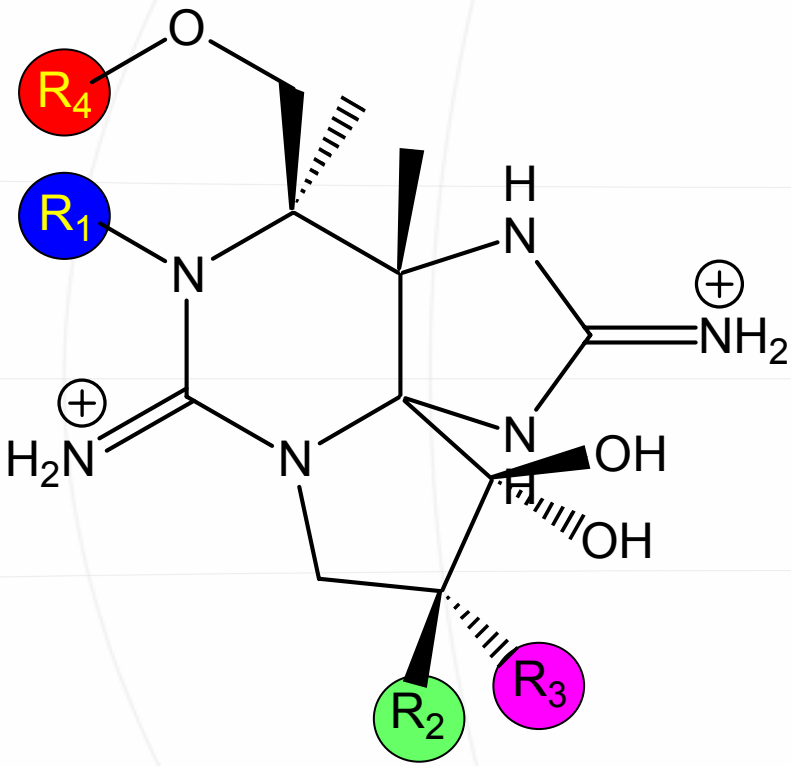
Acetal moiety

Carbamoyl group



caffeine

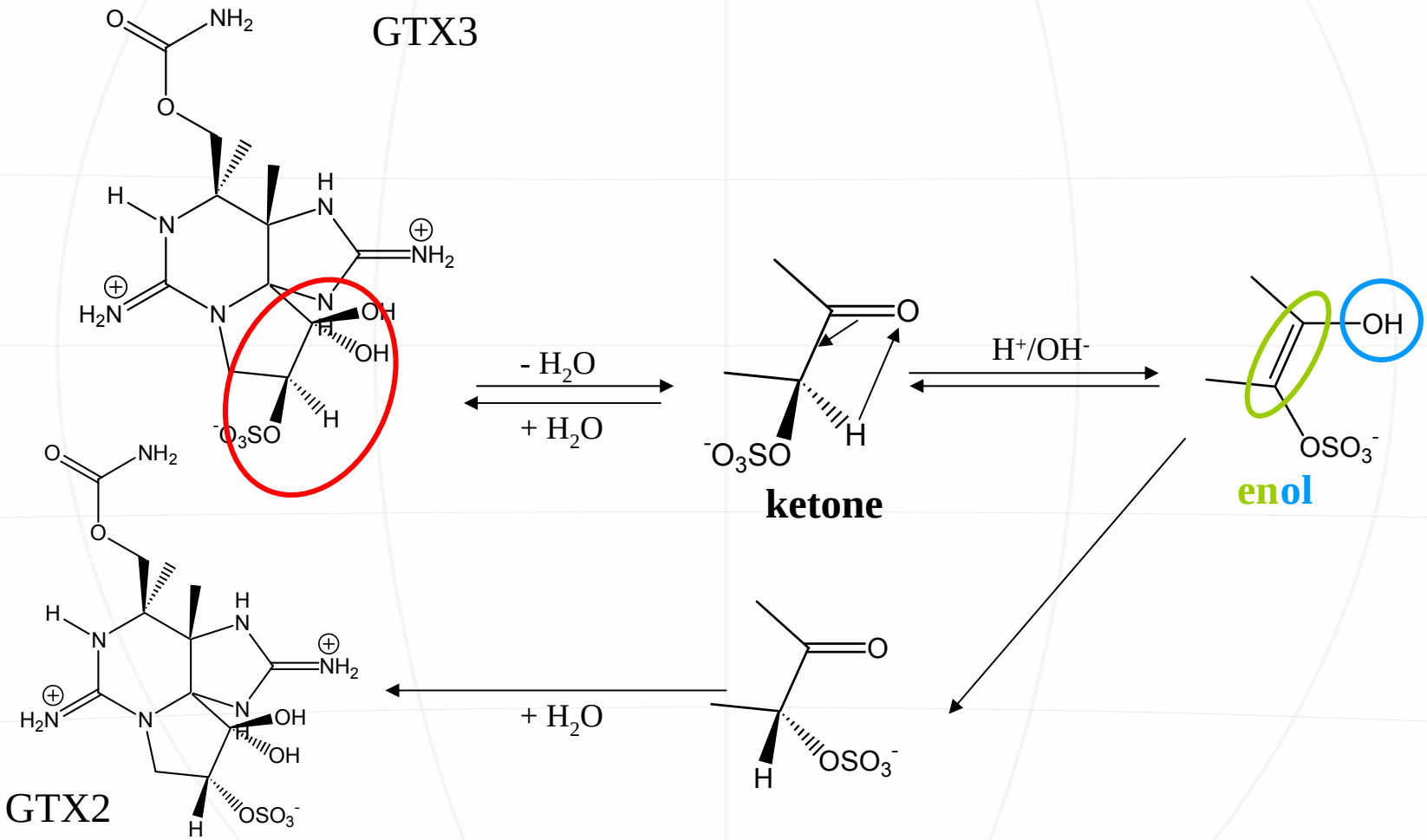
Chemical Structures



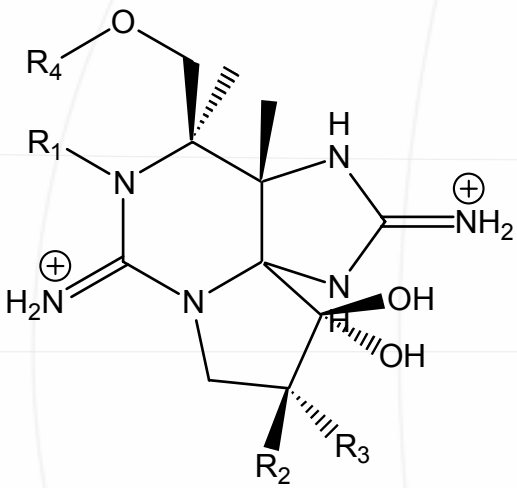
STX = Saxitoxin
NEO = Neosaxitoxin
GTX = Gonyautoxin

Toxin	R1	R2	R3	R4
STX	H	H	H	$CO-NH_2$ (Carbamoyl-)
NEO	OH	H	H	
GTX1	OH	H	OSO_3^-	
GTX2	H	H	OSO_3^-	
GTX3	H	OSO_3^-	H	
GTX4	OH	OSO_3^-	H	
B1= GTX5	H	H	H	$CO-NH-SO_3^-$ (N-Sulfocarbamoyl-)
B2= GTX6	OH	H	H	
C3	OH	H	OSO_3^-	
C1	H	H	OSO_3^-	
C2	H	OSO_3^-	H	
C4	OH	OSO_3^-	H	
dc-STX	H	H	H	H (Decarbamoyl-)
dc-NEO	OH	H	H	
dc-GTX1	OH	H	OSO_3^-	
dc-GTX2	H	H	OSO_3^-	
dc-GTX3	H	OSO_3^-	H	
dc-GTX4	OH	OSO_3^-	H	

11-Sulfate Keto-Enol Tautomery



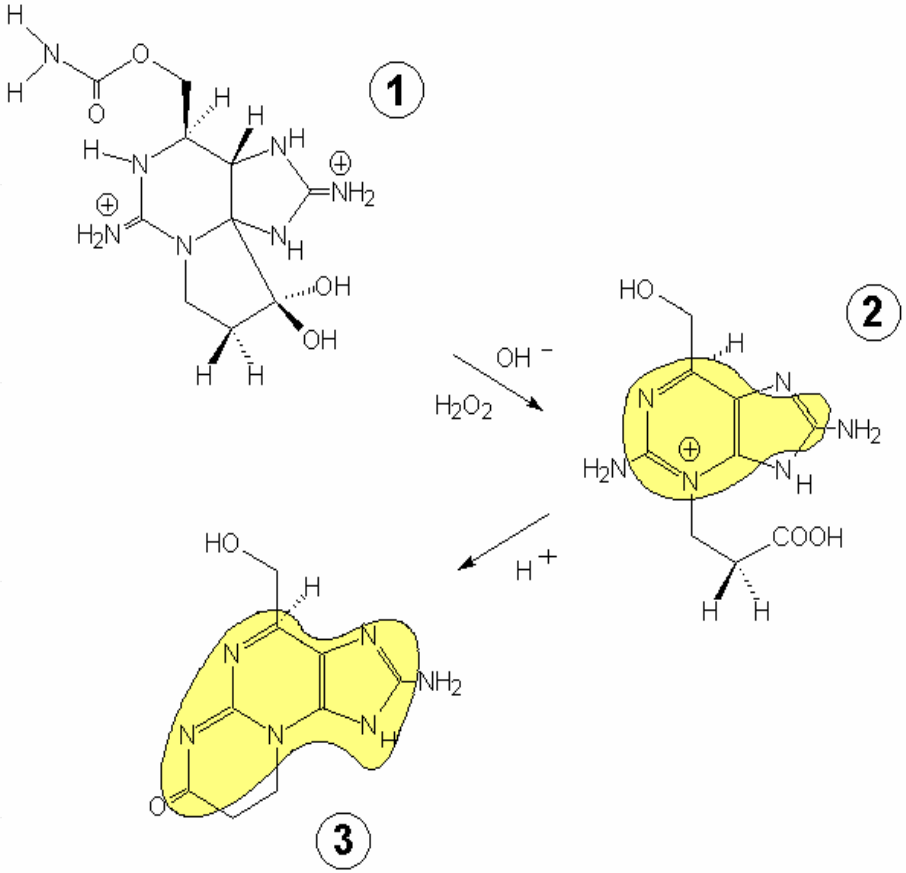
11-Sulfate Keto-Enol Tautomery



The R_2/R_3-toxin isomers are given as sums, because the R_2/R_3 ratios are not stable (except for the equilibrium ratio)

Toxin	R1	R2	R3	R4
STX	H	H	H	$CO\text{-}NH_2$ (Carbamoyl-)
NEO	OH	H	H	
GTX1	OH	H	OSO_3^-	
GTX2	H	H	OSO_3^-	
GTX3	H	OSO_3^-	H	
GTX4	OH	OSO_3^-	H	
B1= GTX5	H	H	H	$CO\text{-}NH\text{-}SO_3^-$ (N-Sulfocarbamoyl-)
B2= GTX6	OH	H	H	
C3	OH	H	OSO_3^-	
C1	H	H	OSO_3^-	
C2	H	OSO_3^-	H	
C4	OH	OSO_3^-	H	
dc-STX	H	H	H	H (Decarbamoyl-)
dc-NEO	OH	H	H	
dc-GTX1	OH	H	OSO_3^-	
dc-GTX2	H	H	OSO_3^-	
dc-GTX3	H	OSO_3^-	H	
dc-GTX4	OH	OSO_3^-	H	

Oxidation of PSP Toxins

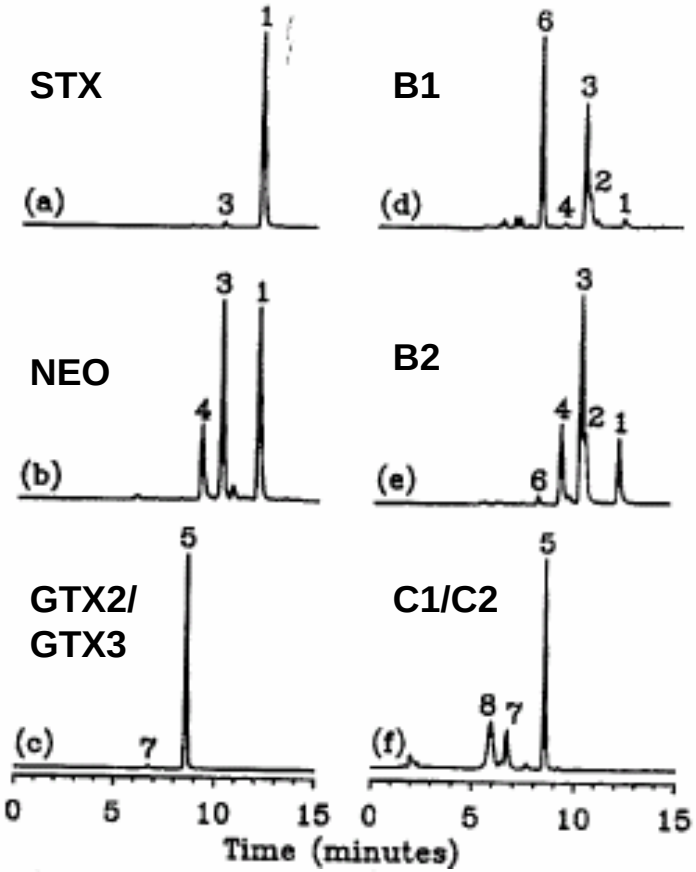


1. PSP toxin eluting from column showing neither UV nor fluorescence activity

2. Oxidation with H_2O_2 or periodic acid

3. Acidifying with acetic acid or nitric acid, products showing strong fluorescence (Ex: 330 nm, Em: 390 nm)

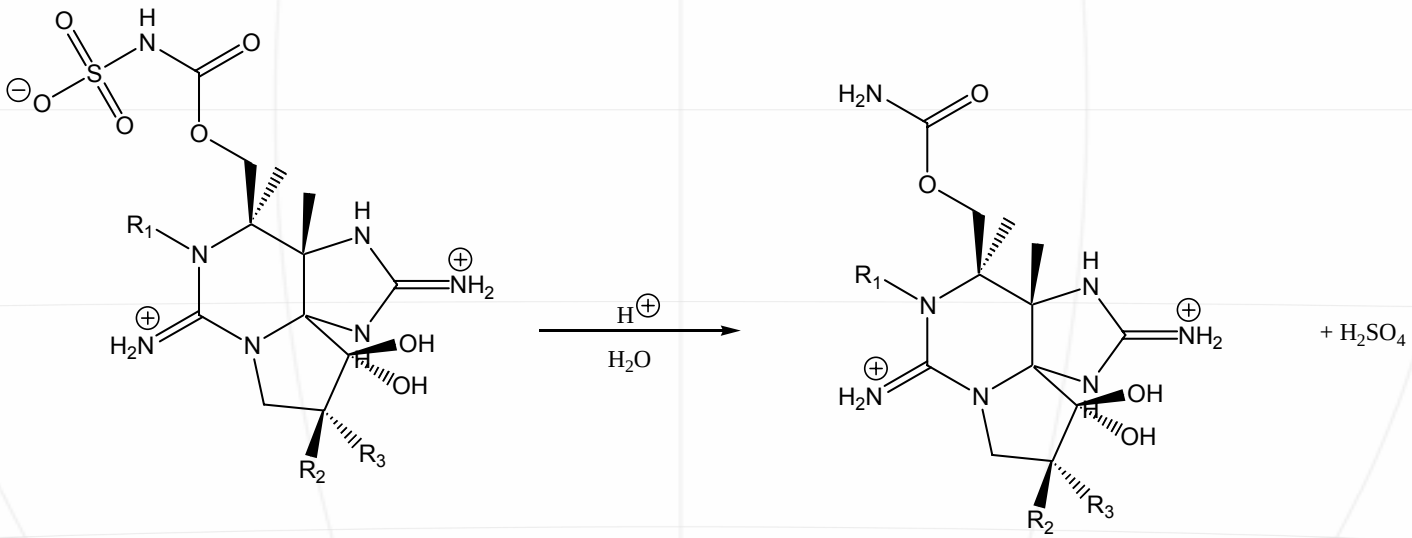
Oxidation of PSP Toxins



Janeček, M. et al. (1993) *J. Chromat.* 644, 321-331

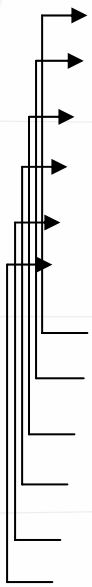
Hydrolysis of B- and C-Toxins

No C-toxin standards are commercially available,
Therefore N-(sulfocarbamoyl) toxins have to be desulfonated into carbamoyl toxins for quantitation



Hydrolysis conditions: 1 N HCl, 15 min, 90°C

Hydrolysis of B- and C-Toxins



Toxin	R1	R2	R3	R4
STX	H	H	H	$CO\text{-}NH_2$ (Carbamoyl-)
NEO	OH	H	H	
GTX1	OH	H	OSO_3^-	
GTX2	H	H	OSO_3^-	
GTX3	H	OSO_3^-	H	
GTX4	OH	OSO_3^-	H	
B1= GTX5	H	H	H	$CO\text{-}NH\text{-}SO_3^-$ (N-Sulfocarbamoyl-)
B2= GTX6	OH	H	H	
C3	OH	H	OSO_3^-	
C1	H	H	OSO_3^-	
C2	H	OSO_3^-	H	
C4	OH	OSO_3^-	H	
dc-STX	H	H	H	H (Decarbamoyl-)
dc-NEO	OH	H	H	
dc-GTX1	OH	H	OSO_3^-	
dc-GTX2	H	H	OSO_3^-	
dc-GTX3	H	OSO_3^-	H	
dc-GTX4	OH	OSO_3^-	H	

B1 -> STX

B2 -> NEO

C3 -> GTX1

C1 -> GTX2

C2 -> GTX3

C4 -> GTX4

Ion pair chromatography with post-column oxidation and fluorescence detection

1. Instrument: Agilent LC1100:
   Degasser G1379A
   Quaternary pump G1311A
   Autosampler G1329A
   Sample thermostat G13308
   FLD G1321A

   Post-column derivatisation: Pickering PCX 2500

2. Flow: 1 mL/min

3. Eluent A: 6 mM octanesulphonic acid
   6 mM heptanesulphonic acid
   40 mM ammonium phosphate
   0,75% THF

4. Eluent B: 13 mM octanesulphonic acid
   50 mM phosphoric acid adjusted to pH 6.9 with ammonia
   15% ACN
   1.5% THF

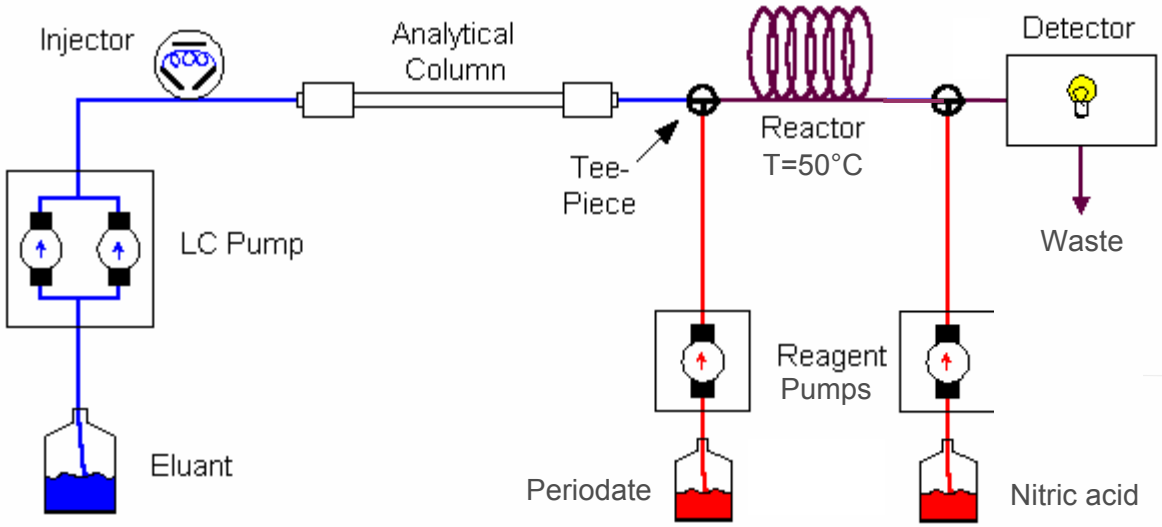
5. Gradient: 0 min 100% A
   15 min 100% A
   16 min 100% B
   35 min 100% B
   36 min 100% A
   45 min 100% A

Ion pair chromatography with post-column oxidation and fluorescence detection

7.	Injection:	20 µL	
8.	Sample thermostat:	4°C	
9.	Precolumn:	Phenomenex SecuriGuard	
10.	Column:	Phenomenex Luna C18, 5µ, 250 x 4.6 mm	
11.	Derivatisation: each 0.4 ml/min	1. 10 mM Periodic acid 550 mM Ammonia 2. 0.75 N Nitric acid	
12.	Reactor temperature:	50°C	
13.	Detection: Fluorescence measuring:	excitation wave length: emission wave length:	333 nm 395 nm

Krock B. et al. (2007) *Harmful Algae.* in press

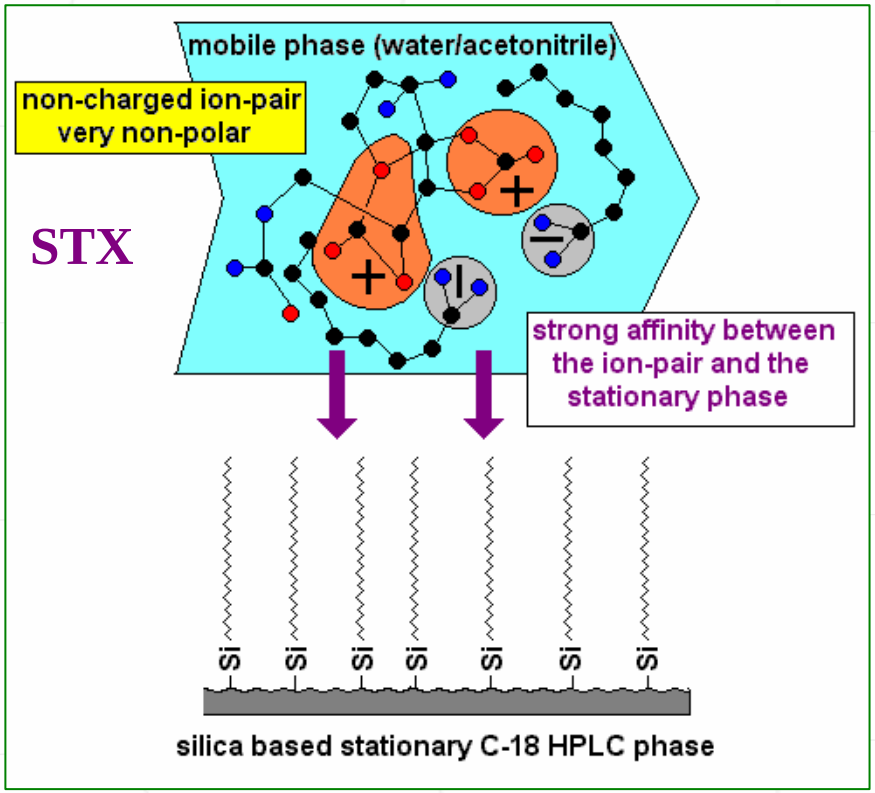
Post-column oxidation of PSP Toxins

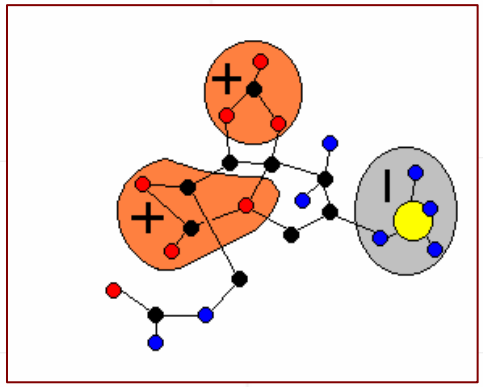


Schematic diagram of a post-column derivatisation system

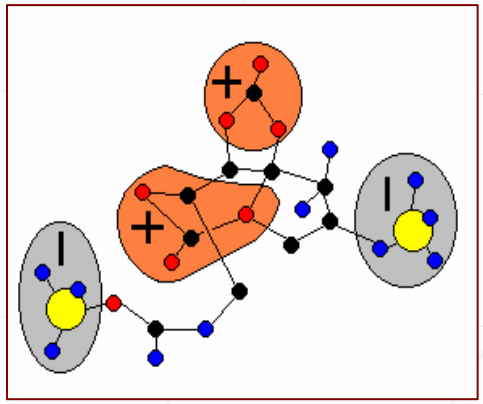
Retention under Ion Pair Conditions

Ion pair chromatography: Organic anions are added to the mobile phase to form neutral complexes ("ion pairs") with kations, or vice versa





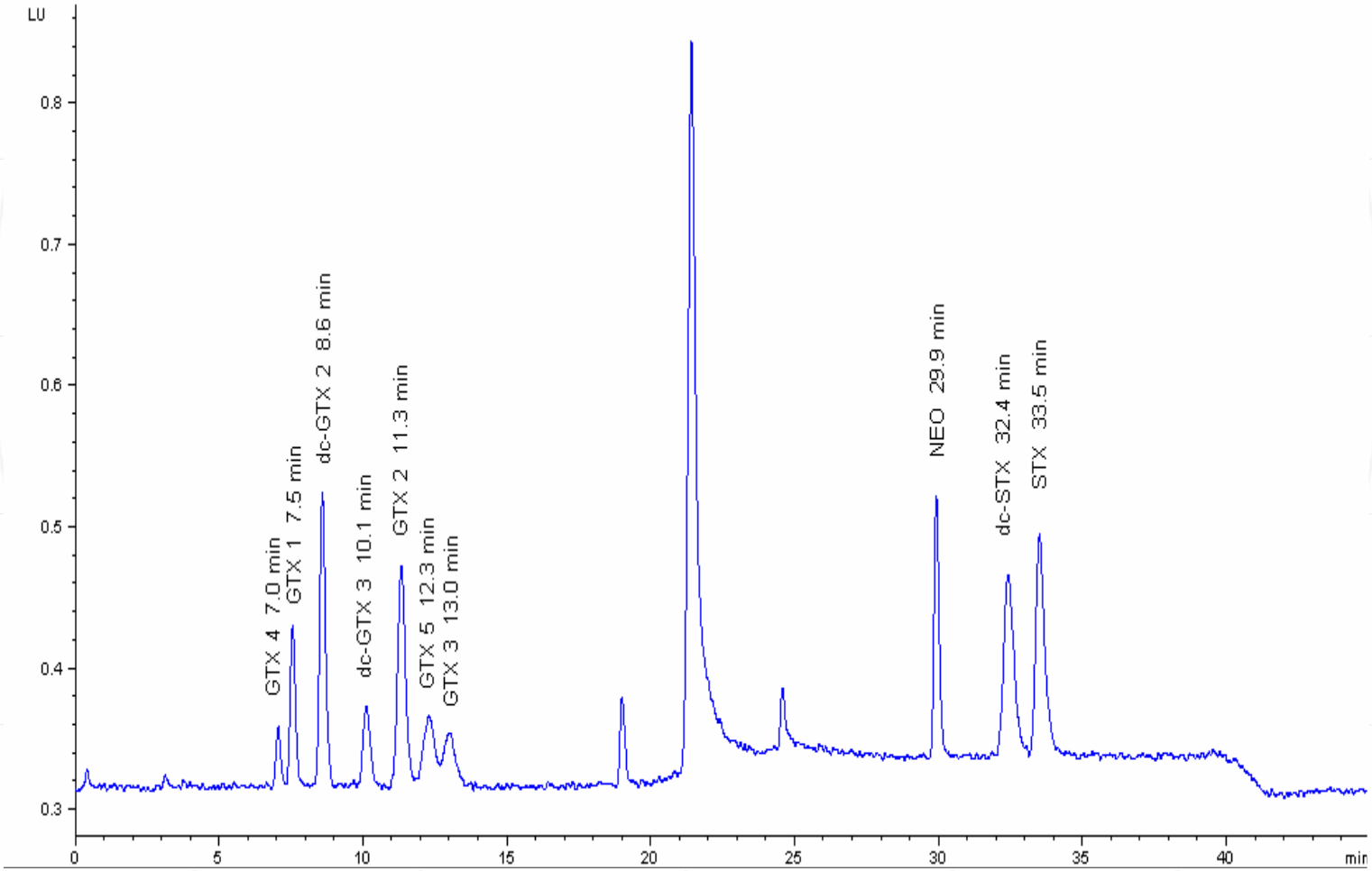
GTX-3



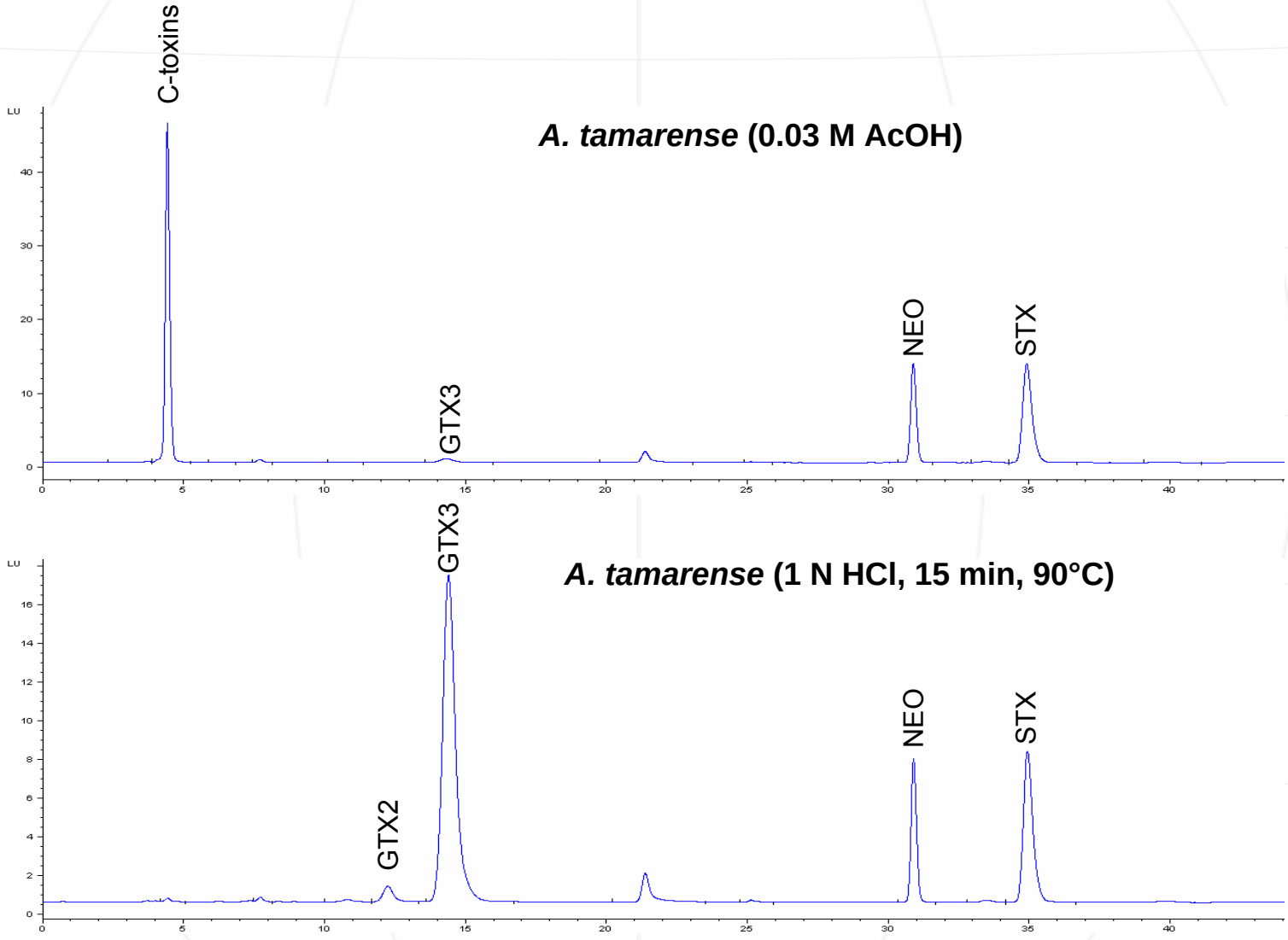
C-2

Retention: C2 < GTX3 < STX

Retention under Ion Pair Conditions



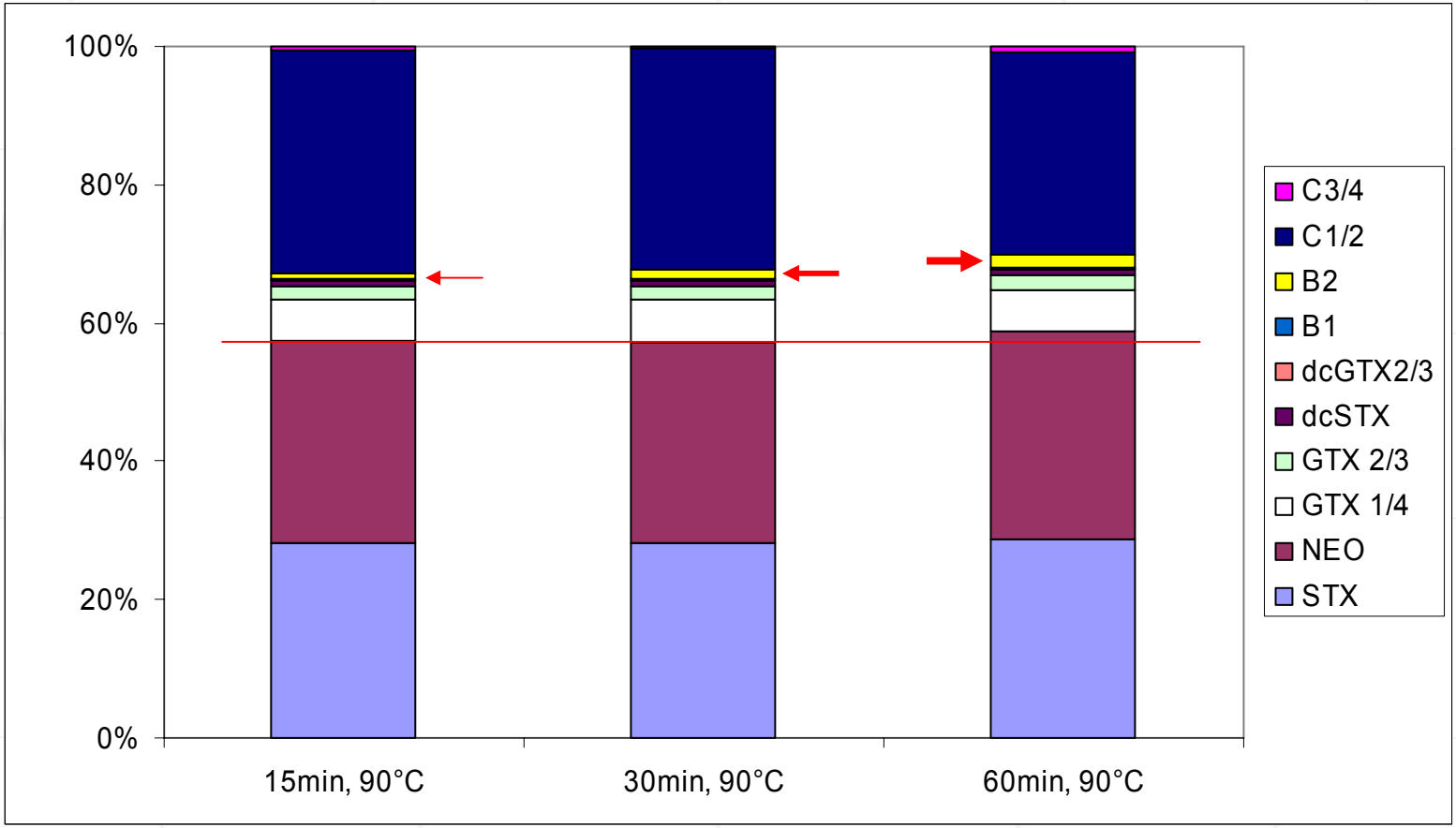
Retention under Ion Pair Conditions



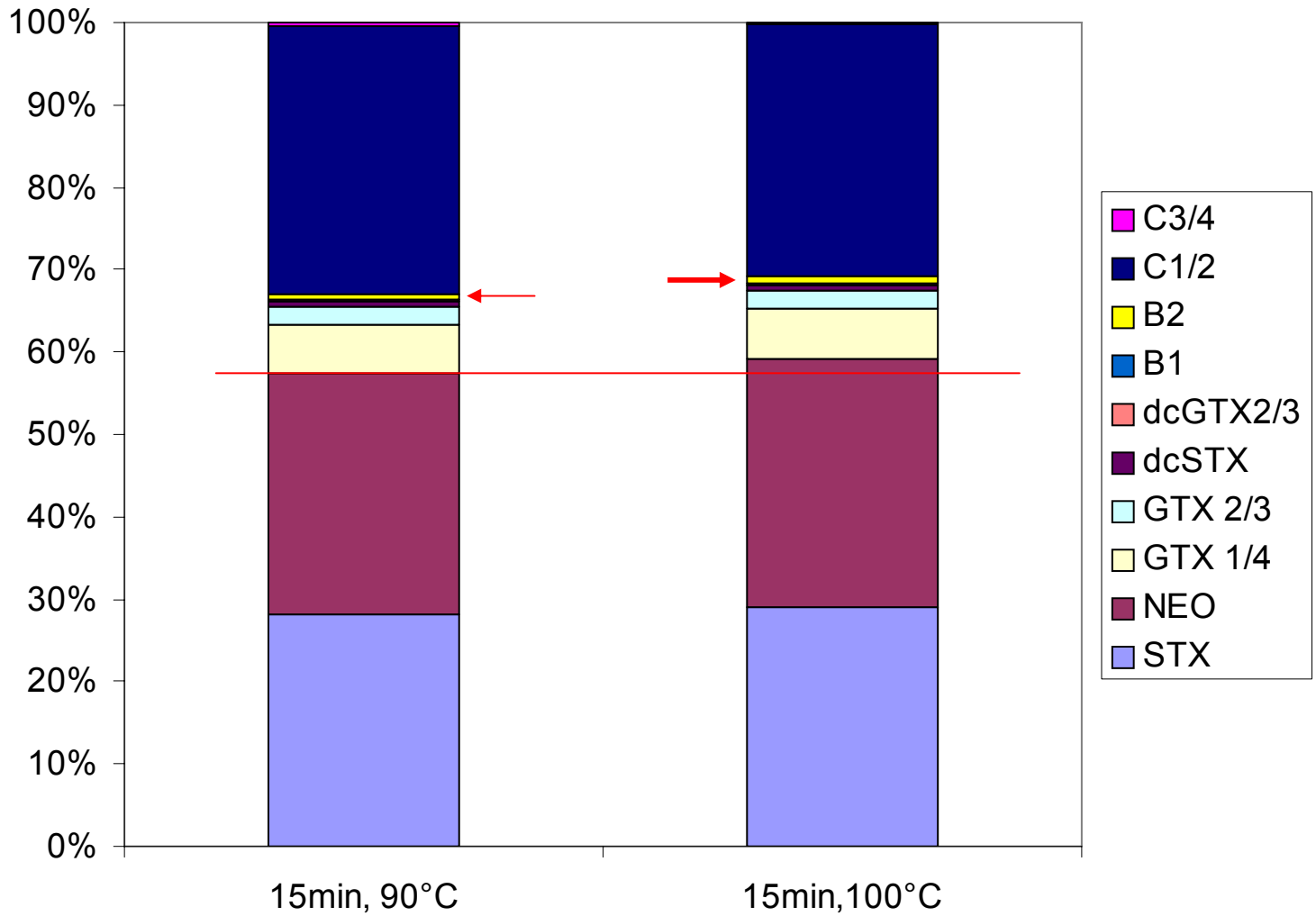
Sensivity

Toxin	HPLC-FLD Limit of Quantitation (LOQ) (S/N=5) [pg]	LC-MS/MS (API 4000 Q-Trap) Limit of Quantitation (LOQ) (S/N=5) [pg]
GTX4	1190	7
GTX1	1571	112
dcGTX2	48	77
dcGTX3	55	51
GTX2	63	95
B1	329	10
GTX3	67	5
NEO	585	49
dcSTX	62	12
STX	61	14

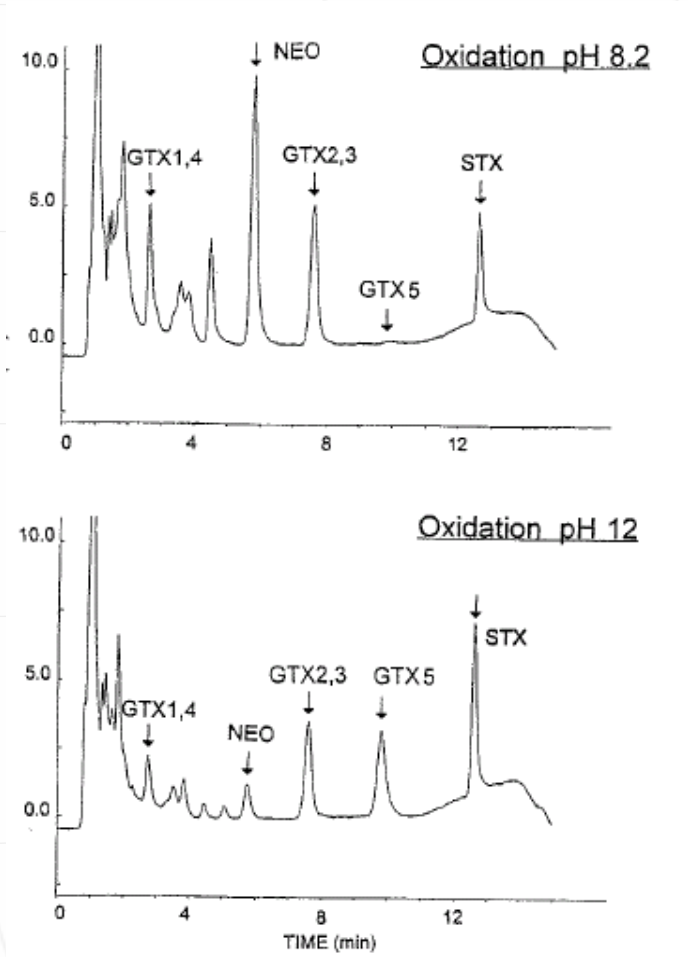
Hydrolysis of B- and C-Toxins: Time



Hydrolysis of B- and C-Toxins: Temperature

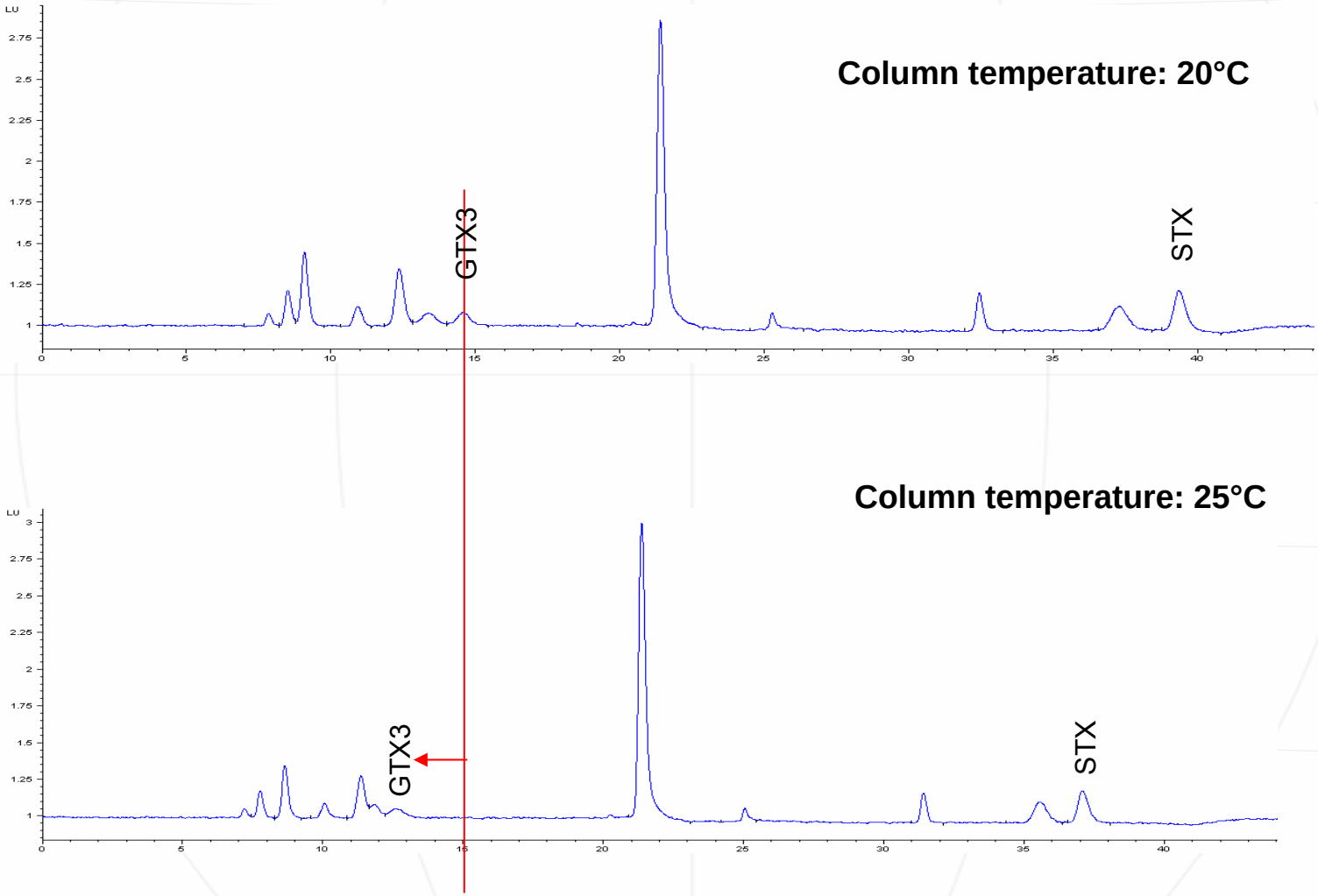


Oxidation: pH

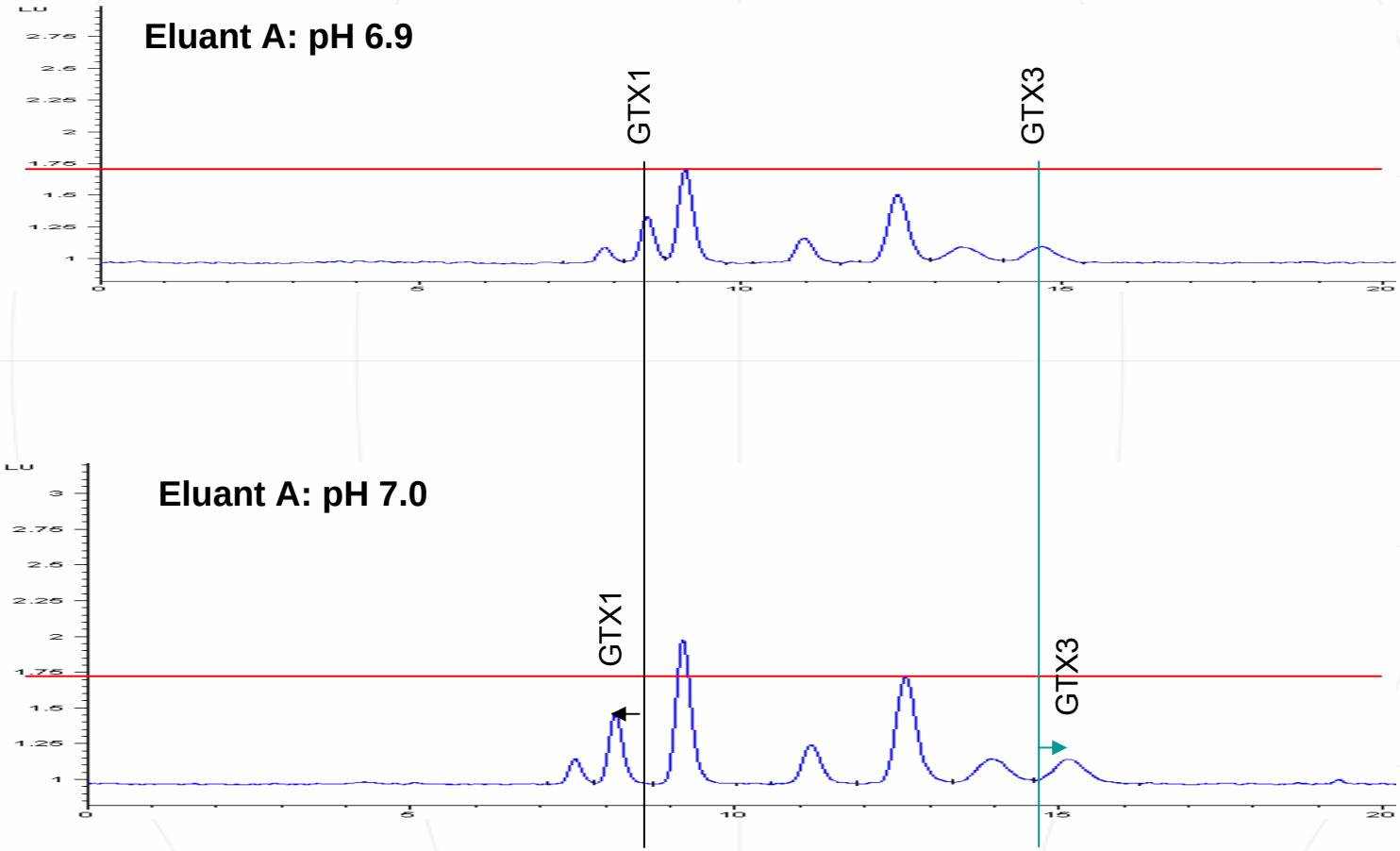


Gago-Martínez, A. et al. (2001) *J. Chromat. A* 905, 351-357

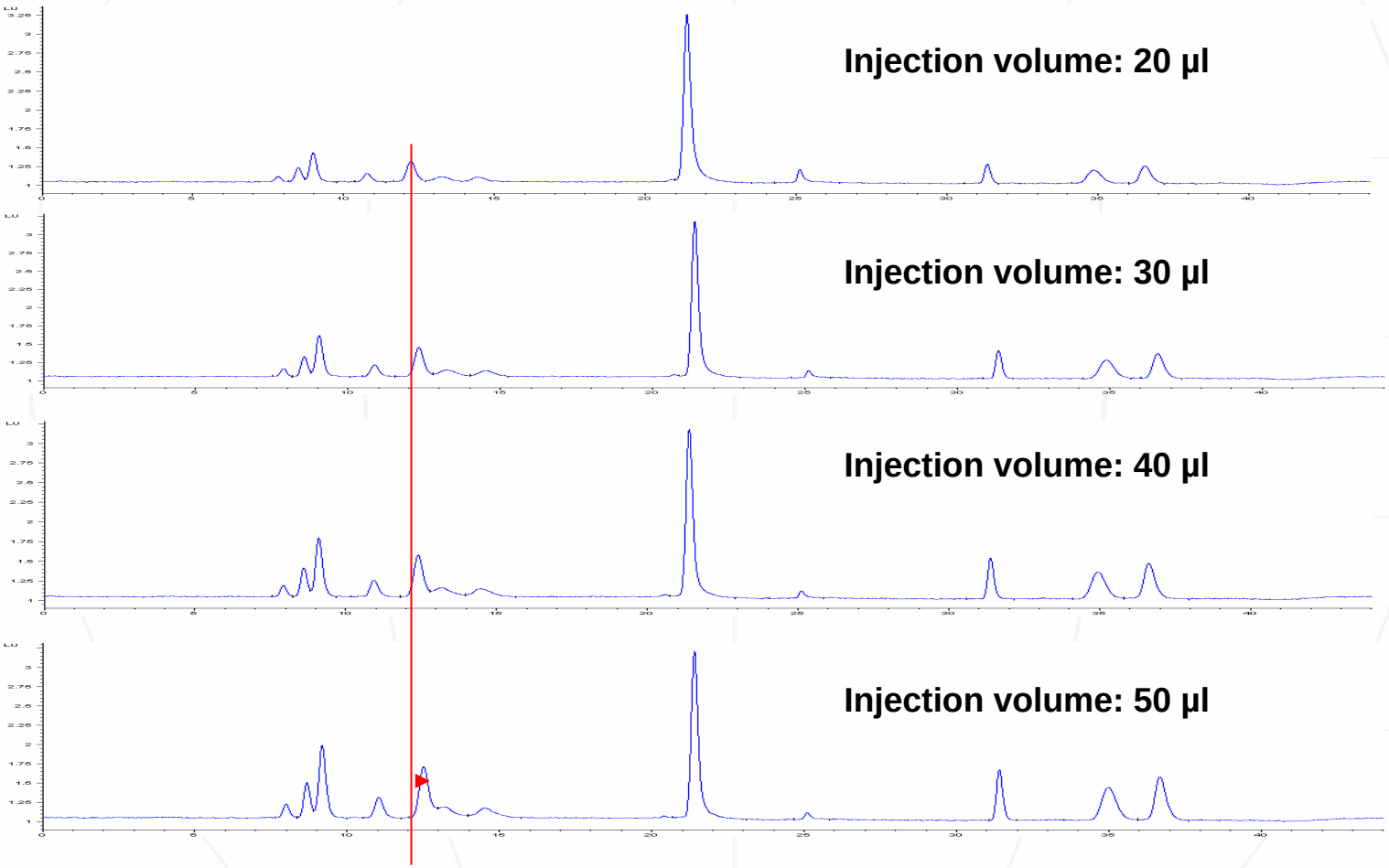
Column: Temperature



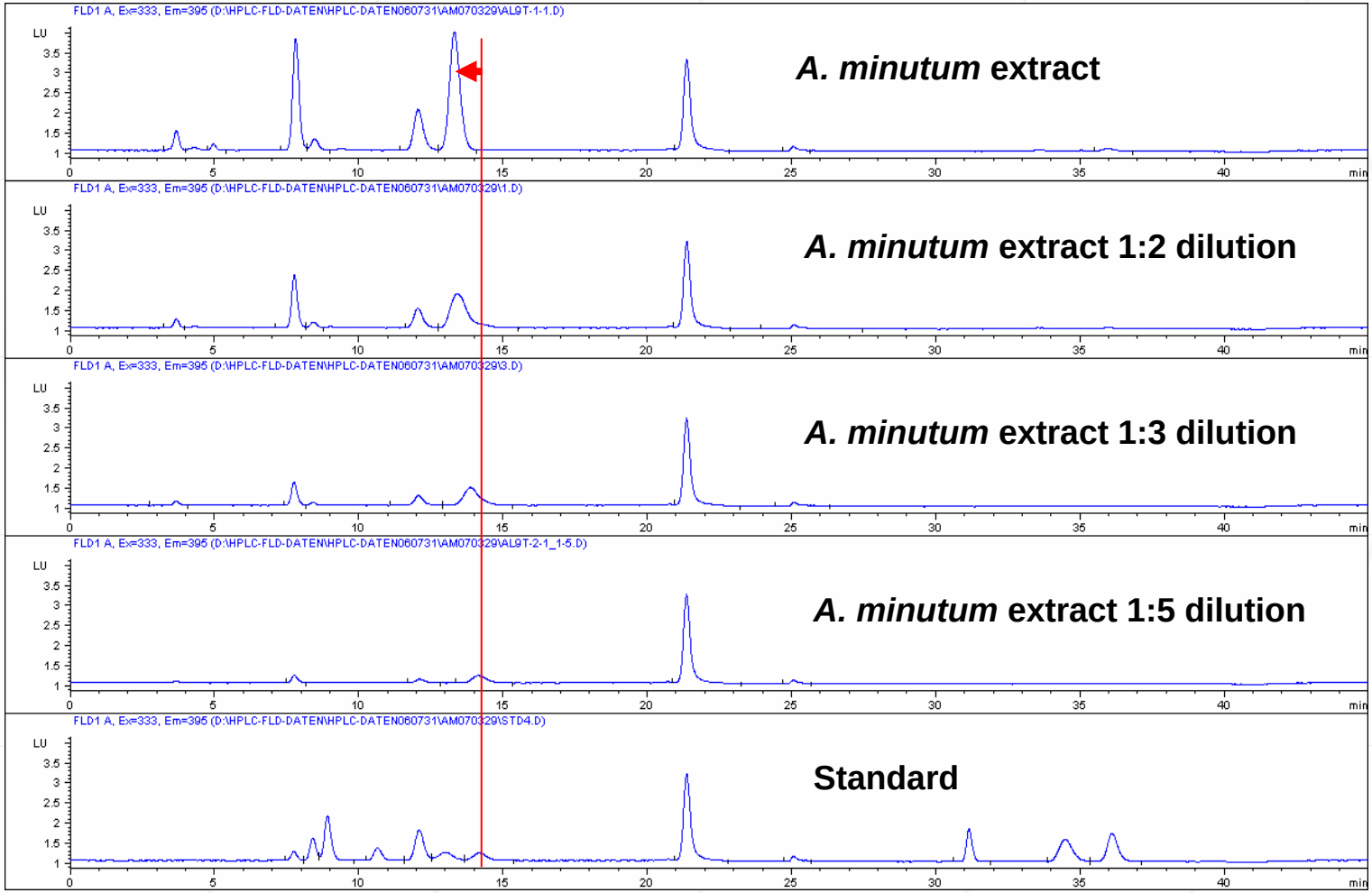
Eluants: pH



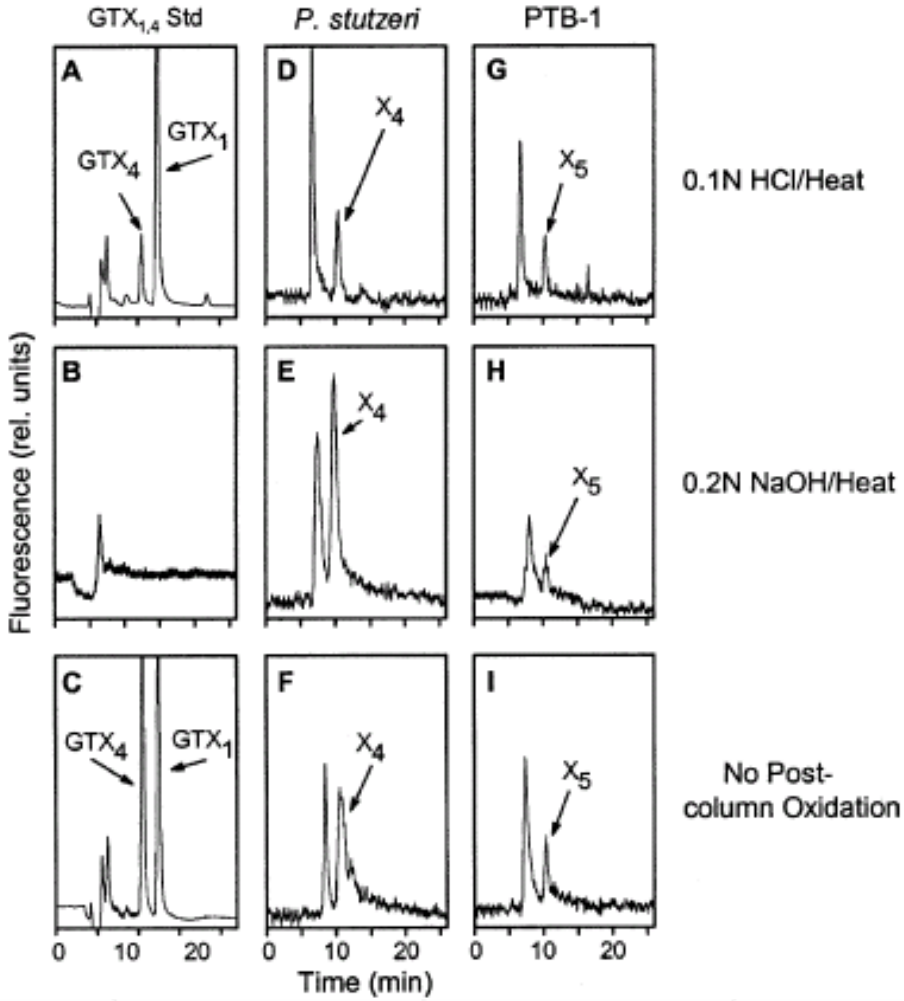
Injection: Volume



Matrix Effects



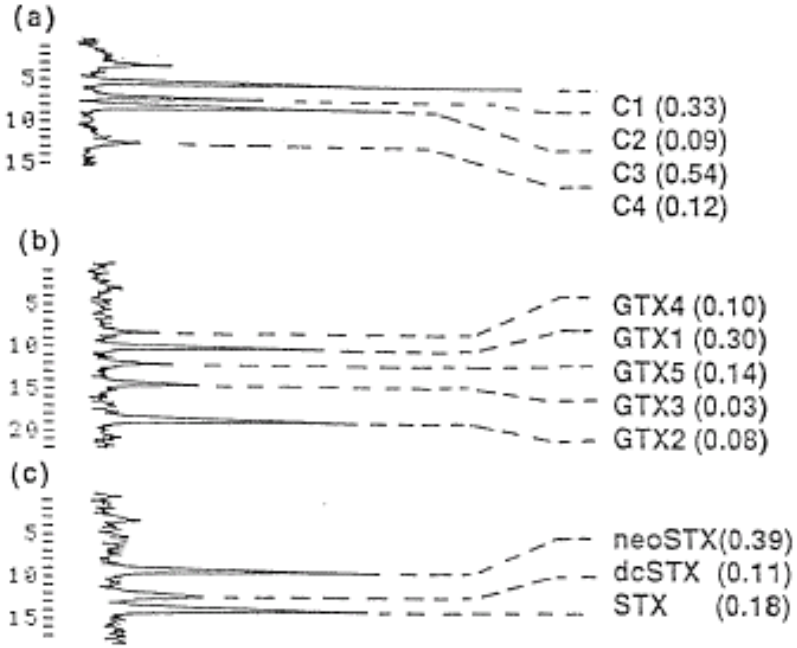
Imposters



Baker T.R. et al. (2003) *Toxicon* 41(3) 339-347.

Oshima method (post-column oxidation)

column Inertsil C8-5, 4.6x150mm
flow 0.8 ml/min



C-group: 1 mM TBAP, adjusted to pH 5.8 with AcOH

GTX-group:2 mM Na 1-heptanesulfonate in 10 mM $(NH_4)_2PO_3$, pH 5.8

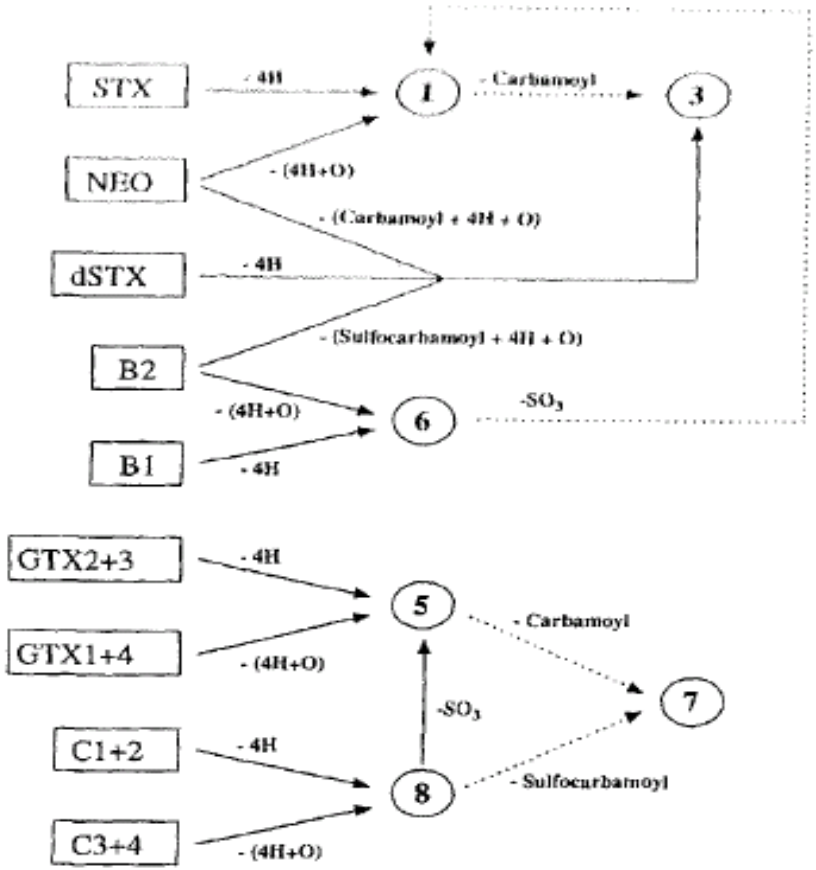
STX-group:2 mM Na 1-heptanesulfonate in 30 mM $(NH_4)_2PO_3$, pH 7.1:ACN = 10:5

Oshima, Y. (1995) *Manual on Harmful Marine Microalgae, IOC Manuals and Guides No. 33*. G. M. Hallegraeff, D. M. Anderson and A. D. Cembella. Paris, UNESCO: 81-94.

Lawrence method (pre-column oxidation)

1. Flow: 100 µL/min
2. Eluent A: 10 mM heptafluoro butyric acid adjusted to pH 4.2 with NH_4OH
3. Eluent B: ACN
4. Gradient: 0 min 100% A
   20 min 80% A
   21 min 100% A
   33 min 100% A
5. Injection: 20 µl
6. Column: LiChrospher-100 RP18, 5µ, 250 x 1.0 mm
7. Derivatisation: 1. 30 mM Periodic acid, 300 mM Na_2HPO_4, 300 mM NH_4HCOO adjusted to pH 9.0 with 1 M NaOH (prepared daily) (1:1:1 v/v/v)
   2. 50% aqueous acetic acid
8. Detection: Fluorescence measuring:
   excitation wave length: 335 nm
   emission wave length: 400 nm

Lawrence method (pre-column oxidation)

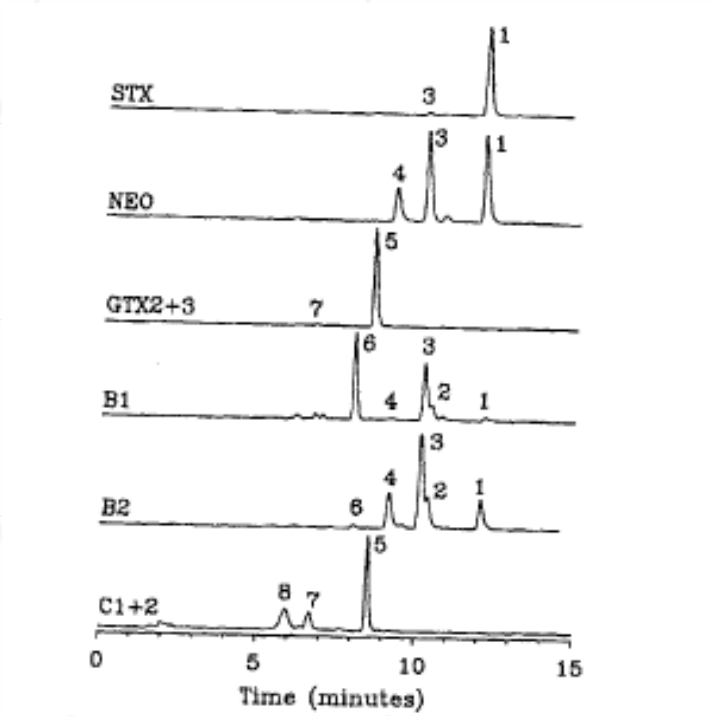


Not validated for decarbamoyl toxins (except for dcSTX)

1-*N*-hydroxylated PSTs have to be purified by 2 subsequent SPE steps

Quilliam M.A. et al. (1993) *Rapid Commun. Mass Spectrom.* 7, 482-487

Lawrence method (pre-column oxidation)



Complex toxin profile cannot be resolved

Quilliam M.A. et al. (1993) *Rapid Commun. Mass Spectrom.* 7, 482-487

1. Ion pair chromatography with post-column oxidation and fluorecence detection is highly selective and sensitive method for PSP determination

2. B- and C- toxins can indirectly be determined by acidic hydrosis

3. 11-sulfate toxins (C1/C2, C3/C4, GTX1/GTX4, GTX2/GTX3, dcGTX1/dcGTX4, dcGTX2/dcGTX3) should be given as sums only due to their easy interconvertability

4. HPLC- and oxidation parameters have to be carefully controlled in order to keep retention times and molecular response constant

5. Despite of high selectivity signals have to be confirmed by experiments or inddependent methods

Thanks to…

Annegret Müller, AWI

…and for your attention!