AD_____

ANALYSIS OF SAXITOXIN FROM URINE USING DYNAMIC FAB/MS

MIDTERM REPORT

CHESTER J. MIROCHA
WON JO CHEONG
HAMED ABBAS

JULY 31, 1990

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-89-C-9101

University of Minnesota
1919 University Avenue
St. Paul, Minnesota 55104

SECURITY CLASSIFICATION OF THIS PAGE

AD-A226 474

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Minnesota 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 1919 University Avenue St. Paul, Minnesota 55104	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-89-C-9101
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21702-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 0603002A PROJECT NO. 3M2-63002D807 TASK NO. AH WORK UNIT ACCESSION NO. 052

11. TITLE (Include Security Classification)
(U) Analysis of Saxitoxin from Urine Using Dynamic FAB/MS

12. PERSONAL AUTHOR(S)
Chester J. Mirocha, Won Jo Cheong, and Hamed Abbas

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Midterm	FROM 7/15/89 TO 7/14/90	1990 July 31	83

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	11		RA 1, BD, Lab Animals; Guinea pigs, Rats, Saxitoxin, Dynamic FAB, Analysis, Mass Spectrometer
06	04		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Analytical procedures for the analysis of saxitoxin (STX) have been investigated using Continuous Flow Fast Atom Bombardment (CFFAB). Items investigated included derivative formation of STX resolution by micro capillary open tube columns (25 to 100 microns i.d.), CFFAB matrices, solvent flow rates, probe tip design and selection of fragment ions for analysis by Selected Ion Recording. Five-hundred picograms of STX can be reproducibly detected by SIR using a matrix made up of 5% thioglycerol, 1% acetic acid and 0.05% sodium dodecyl sulfate.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

TABLE OF CONTENTS

TABLE OF CONTENTS

I. INTRODUCTION

Saxitoxin was first isolated and described from toxic Alaska butter clams known by the Latin binomial **Saxidomus giganteus** [1]. Paralytic shellfish poison (PSP) is one of the most notorious marine toxins known [2]. PSP, once ingested by humans, evokes paralysis and other symptoms, with frequent death. It is estimated that the number of victims of paralytic shellfish poisoning worldwide between 1972-1983 exceeded 900, including 40 deaths [2]. Sommer et al. [3] elucidated the course of events leading to PS poisoning: **Protogonyulax** (formerly **Gonyaulax**) catenella produces PSP, and grows up to a high density under favorable environmental conditions; the organism then infests bivalves which in turn may poison humans when ingested. Later **P.tamarensis, Pyrodinium bahamense, Pyr. bahamense, Var. compressa, Aphanizomeno flos-aquae**, etc. were added to the list of PSP-producers.

There have been a number of studies on occurrence, chemistry, structure, and toxicity of STX and related shellfish toxins [1,4- 15]. Schantz et al. [4] first determined the correct structure of STX (See Figure 1). According to Schantz [6], STX is a basic substance forming salts with mineral acids. The hydrochloride is hygroscopic, a white solid crystal, very soluble in water, sparingly soluble in methanol and ethanol, and

insoluble in lipid solvents. Two basic functions (guanidine moities) are associated with the molecule, one at pKa 8.3, and the other at pKa 11.5. The HCl salt is stable to the temperature of boiling water at pH 5 or less but is destroyed in alkaline solutions. Kish et al [15] first synthesized STX in 1977.

Various analytical methods for saxitoxin [16-27] have been reported, and among these, a well-established technique is HPLC separation followed by postcolumn fluorescence detection [18-20,22,23]. Using a polystyrene divinylbenzene resin column and alkaline periodic acid as the postcolumn fluorescence reagent, Sullivan et al. [18] were able to detect 100pg STX. Wright et al. [27] have recently reported a new technique for STX: Separation by Capillary Zone Electrophoresis(CZE) followed by laser fluorescence detection. They were able to detect 50pg STX with a good signal to noise ratio(>5).

Development of Fast Atom Bombardment(FAB) technique enabled analysis of STX by Mass Spectrometry [16,17]. Nevertheless quantitative determination of STX using FAB has not been reported, and only mass spectra of STX was measured using rather a large amount of STX (1-10ug). On the other hand, Quilliam et al. [26] reported a nice mass spectrum of STX measured by Ion-Spray Mass Spectrometry (ISMS) using 100ng STX and determined a very low Selective Ion Monitoring (SIR) detection

limit of STX (30pg). Their study was carried out by flow injection of pure saxitoxin; thus there has been no report on the quantitative analysis of STX by a proper LC/MS technique.

The ultimate goal of this project is to establish an analytical technique by which a low level STX (500pg or less) can be quantitatively and reproducibly separated and detected by Mass Spectrometry. The study of the first year has been focused on improvements in MS techniques. In most of our LC/MS studies, an open tubular silica capillary was used, and STX was injected into the capillary by an injector with a 500nl internal sample loop. In development of separation or purification of STX, only a preliminary study has been carried out.

In this report, we describe improvements in FAB/MS analysis , then cooperative studies with other groups using different mass spectrometer instruments, followed by various trials of derivatization of STX (feasibility study of GC/MS), preliminary study of separation (LC technique) and purification of STX. Finally future research plans for the second year will be presented.

II. CONTINUOUS FLOW FAST ATOM BOMBARDMENT STUDY.

There have been only a few reports in the literature on the

detection of STX (saxitoxin) by FAB/MS. The only available data found in the literature was obtained by the technique using glycerol as the matrix [6,17]. The mass of the molecular ion ([MH]+) is 300 and the mass of a major fragment is m/z 282 which corresponds to the dehydrated molecular ion. The ion of mass 374([MH+glycerol-H2O]+1) is also one of the major peaks. Saxitoxin was also detected in our laboratory using mixtures of water with glycerol or thioglycerol.

The use of Continuous Flow FAB (CFFAB) provides some significant advantages over the standard FAB techniques, including higher sensitivity and lower limit of detection due to the decreased background chemical noise and decreased ion suppression effects [28-32]. In addition, it provides a means for the rapid analysis of large number of samples present in aqueous solutions as well as those dissolved in volatile organic solvents such as acetonitrile or methanol. HPLC/ CFFAB technique has been successfully demonstrated for the analysis of mixtures of peptides and proteins [31-33]. On the other hand no such technique (HPLC/ CFFAB) has been known for PSP toxins although HPLC/ Fluorescence techniques exist. Part of this is because PSP toxins have lower FAB sensitivity and standard FAB matrices cannot serve as HPLC solvents for STX separation by HPLC.

We have improved FAB methodology and achieved rather

reproducible detection of 500pg STX using carefully developed glycerol matrices containing a small amount of surfactant. At the present stage of development, we have been mostly using an open tubular silica capillary (25 microns i.d., 2m, deactivated). Resolution by packed micro columns or bonded phase capillary have been attempted by our laboratory but this area needs more development . In this chapter, we will describe the course of improvement of the CFFAB method and related informative findings in FAB fluid dynamics, effects of various factors on FAB performance, and preferred direction of future studies.

[1]. Mass Spectra of STX and Neo-STX by CFFAB

In CFFAB a smaller quantity of STX is needed than in conventional direct probe FAB in order to get a good mass spectrum. Part of this is due to the better resolution of the components of a mixture forming a tighter packet of molecules flowing across the probe tip and the other is that the matrix background peaks can be eliminated by background subtraction. For example, compare Figure 2 (10 ug STX analyzed by direct probe FAB) and Figure 3 (0.5 ug STX analyzed by CFFAB in thioglycerol) where the the background was subtracted. The molecular ion of STX is 300; m/z 282 represents the molecular ion minus water; m/z 390 is the saxitoxin/thioglycerol adduct minus water. The amount of sample needed in CFFAB is about

50ng. Detection of STX and Neo-STX (50ng each) and the background subtracted FAB mass spectrum of Neo-STX are shown in Figure 4. Neo-saxitoxin is like saxitoxin except for addition of a hydroxyl on the ring nitrogen adjacent to the carbamate nitrogen (Figure 5). Mass m/z 316 represents the molecular ion; m/z 298 the molecular ion minus water; m/z 406 the neosaxitoxin/thioglycerol adduct minus water; m/z 300 the protonated STX formed from Neo-STX; and m/z 282, the protonated STX minus water.

We have observed that STX not only has relatively low FAB activity (compared to peptides) but it has a very low tendency to fragment in the mass spectrometer causing difficulties in MS/MS analysis. In the daughter spectrum (Figure 6) of the STX molecular ion, the parent (molecular ion) is the base peak, and the molecular ion minus water is a low intensity fragment of little significance. This caused us to delay the Multiple Reaction Monitoring (MRM) approach that we initially planned to focus on and turn to Selective Ion Recording (SIR) technique.

[2]. Development of CFFAB Methoddology

We have improved sensitivity of CFFAB through a series of progressive experiments in which column size, matrix, matrix flow and choice of detergent and acid were tested. In order to minimize matrix background we chose to use a higher

instrument resolution; 1500 resolution in SIR mode has been used throughout the work. A Shimadzu LC-600 pump was used to obtain low flow rates (1-10 ul/min). A 25 or 50 micron i.d. deactivated open tubular silica capillaries were used and STX samples were injected by a 0.5 ul Rheodyne injector. First, we cleaned the Xe FAB gun unit and replaced worn-out parts and reassembled the unit paying special attention to internal alignment. The sensitivity was about 10 times better than it had been. Additional ten fold improvement in sensitivity was obtained by addition of a surfactant (sodium dodecyl sulfate). Still additional improvement in sensitivity was achieved by optimizing matrix composition. Reproducibility was also improved by optimizing matrix composition, flow rate, probe tip temperature, and shape of the probe tip.

The original matrix used in our experiments was composed of 1% thioglycerol, 1% acetic acid and water. We improved performance by changing the matrix to 5% thioglycerol, 1% acetic acid, 0.05% sodium dodecyl sulfate and water. Further experimentation revealed that 5% glycerol, 0.5% acetic acid, 0.1% PEG(polyethylene glycol)400, 0.5% PEG300, 0.025% sodium dodecyl sulfate gave us superior results. The PEG is used as an internal calibrant. We are still optimizing the matrix in view of its surfactant type and concentration.

[3]. Factors of CFFAB

Addition of Surfactant:

The dramatic enhancement of sensitivity upon addition of 0.05% sodium dodecyl sulfate is shown in Figure 7. The peak width was narrowed to 1.5 min. and the base line to near 5%.

Matrix Composition:

A matrix with too little thioglycerol (1%) has low viscosity and showed a problem with an unstable liquid surface. Using 5% thioglycerol we were able to improve the chromatographic peak in SIR in addition to improved sensitivity (2-3 times). This effect is shown in Figure 8 by comparing results of 1% and 5% thioglycerol. The improved sensitivity of the new matrix (5%thioglycerol, 1% acetic acid, and 0.05% sodium dodecyl sulfate) in detecting 500pg STX is shown in Figure 9.

Fluid Dynamics:

The input of matrix flow to the tip should be balanced with the matrix removal from the tip in order to obtain a stable thin film matrix for an extended period of time (30 minutes at least). The mechanism of matrix removal is exclusively evaporation, and the evaporation rate is governed by the temperature of the probe tip and the tip geometry. The matrix

should be viscous enough to maintain a stable, smooth surface, but not too viscous to prevent free mixing on the tip. Flow rate and composition of the matrix, temperature and shape of the probe tip are important factors that govern the fluid dynamics and mass balance on the tip. Components of high boiling point will be accumulated and it takes time to reach a dynamic equilibrium in composition and temperature of the matrix at the tip. Thus "conditioning" of the tip is required. In many cases, the volume of the tip liquid keeps increasing and dilution effect due to this phenomenon is frequently observed together with a memory effect (tailing). This problem is illustrated in Figure 10.

In order to avoid these anomalies, gradual removal of the eluant from the tip is necessary. This will also help form a stable thin matrix at the tip and maintain conditions suitable for attaining better sensitivity and reproducibility.

Temperature Effect:

In general the optimum temperature of the FAB source is 50-60 C depending on matrix. We examined effects of higher source temperature on peak shape, stability and sensitivity. At a high source temperature (60-70 C), evaporation of the matrix on the probe tip is faster and therefore the flow of the eluant should be increased to maintain a continuously wet surface at the tip. However, when the flow is too high, the source pressure reaches

its limit and termination of the operation will occur. We increased the source temperature to 70 C, and the flow of the eluant to 5-8 ul/min using our Shimadzu HPLC pump. This flow rate is about the limit of operation of our instrument (VG 7070EQ). Only one of four replicate injections was successfully detected. the peak width of the detected peak was a lot narrower. It is clear that the liquid surface on the probe tip is very unstable at high temperature even though the peak band width is dramatically improved if detected. It should be noted that the probe tip temperature can be even higher than the source temperature because the tip is bombarded by the hot Xe beam. The tip temperature is not monitored directly, so we do not know the exact temperature.

We changed source temperature to 60 C, and the eluant flow rate to 3-4 ul/min.. The results are shown in Figure 11 for repetitive injections of samples with different concentrations. We obtained better peak shape and bandwidth. The reproducability for a 5ng sample is good while the reproducibility for samples of lower concentration is unsatisfactory. We believe that this problem is also related to the unstable matrix surface and/or memory effect. Use of a frit tip may be a solution to that.

The Selected Matrix:

We have chosen the new matrix using the following criteria: 1). The matrix should not include thioglycerol because it deteriorates on the probe tip and causes tar formation. Thus, the tip can be used for a prolonged period. 2). The matrix should include polyethylene glycol which provides nice reference peaks (every 44 mass units), which enables easy internal calibration for various analytes. 3). The matrix should have enough viscosity so that it spreads uniformly over the surface of the FAB tip probe. 4). The amount of the surfactant (sodium dodecyl sulfate) is kept to a minimum (0.025%). If concentrated, the surfactant accumulates on the tip and makes the tip matrix too viscous. As the content of the surfactant is minimized, the tip can be cleaned less frequently.

Selection of an optimum temperature of the tip for the detection of an analyte is a function of both the matrix composition and flow rate. Temperature is the major factor that governs the evaporation rate on the tip and hence the evaporation rate should be balanced with the inflow of the matrix. The flow rate and the tip temperature determine the actual liquid film composition and thickness on the tip at their dynamic equilibrium. For the selected matrix and a flow rate of 8ul/min., the optimum temperature has proven to be 55C as shown in Figure 12. It should be noted that the equilibrium is not perfect even at 55C, and a gradual decrease of sensitivity (gradual increase of the film thickness) is often observed (See

Figure 12-B). Figure 12-C indicates that 60 C is too high for effective film formation; mostly likely the film at the surface dries too quickly. It should be noted that the actual temperature of the probe tip cannot be measured and the reported temperatures are only approximations based on the temperature around the heater element.

The composition of the "selected matrix" is: 5% glycerol, 0.5% acetic acid, 0.025% NaDOS, 0.1% PEG400, 0.5% PEG300. It was discovered by trial and error. Its performance is shown in Figure 13 in comparison with two other matrices as described in the figure caption. The selected matrix gives reproducible peaks representing multiple injections of 500 picograms every three minutes through a 25 micron i.d. open bore fused silica capillary (Fig.13C). The selected matrix clearly shows much better performance than others although there may be other matrices not yet experimented with that could give equal or superior results. It appears that each matrix has its own optimum conditions (flow rate, tip temperature, shape of the tip etc.) for analyses.

EFFECT OF CONDITIONING:

The tip surface of the CFFAB probe tends to become contaminated with tar-like products after a prolonged use especially when thioglycerol or a biological matrix is used. .

The metal tip needs a conditioning procedure as follows: 1) Cleaning with 1N HCl solution. 2) Cleaning with methanol. 3) Rinsing with the matrix at 5ul/min. for ca. 30 minutes. These steps are necessary to make the matrix spread uniformly over the tip. The effect of conditioning the tip is demonstrated in Figure 14 A&B. Note that the initial sensitivity of the unconditioned tip (trace B) is better than sensitivity of the conditioned tip (trace A) although the sensitivity quickly vanishes. The unconditioned tip surface can initially produce a very thin liquid film and give a very high sensitivity, but the film is unstable and collapses quickly. On the other hand, the conditioned tip can produce a thicker but stable film and give a less sensitive but reproducible signal.

EFFECT OF TIP SHAPE:

We have examined how the shape of the tip affects reproducibility and sensitivity (See Figure 15 for geometry and different tip shapes). For the selected matrix, the flat tip gives the best results as shown in Figure 16. The poor reproducibility and poor geometry of the peak obtained with the round tip is due to the fact that the tip is almost dry at the center. For the tapered tip, the liquid at the tip does not smoothly flow along the tapered line as we expected, but it forms an unstable thick drop and gives poor sensitivity as well as poor reproducibility. We project that a flat tip coupled to

a frit may be the most functional.

MATRIX FLOW RATE:

We have found that for our system (the selected matrix and the flat tip at 55C), the optimum flow rate is ca. 5ul/min. When flow rate is too slow (less than 3ul/min.), the tip tends to dry. When flow rate is too fast (higher than 8ul/min.), it is difficult to maintain a stable liquid film on the tip, and the source pressure becomes too high to operate. In general, as the flow rate increases, the peak width narrows. We can see this phenomenon in Figure 17 where two flow rates (5ul/min vs. 8ul/min) are compared. The reproducibility between injections in A is superior to that in B; however, the peak shape in B is much better and hence the sensitivity would be greater than that for A. The peak height in B falls off slightly with repeated injections.

USE OF ADSORBENT:

We used a ring-shaped sponge adsorbent between the metal tip and the plastic insulator of the probe hoping it would facilitate a smooth matrix flow; however, it was observed that the matrix could not reach the adsorbent at all. We also tried an adsorbent pad attached in a subsidiary probe that is perpendicular to the CFFAB probe. The pad was allowed to touch

the CFFAB tip by the edge. Unfortunately, geometric restrictions prevented it from making a good contact.

NEGATIVE ION MODE CFFAB:

We attempted detection of saxitoxin in the negative FAB mode using the selected matrix to examine if its sensitivity is better than that of positive ionization FAB mode. Negative ionization FAB was not effective as no anion peak was detected ; however, the surfactant anion (dodecyl sulfate) was prominent.

EFFECT OF SURFACTANT CHAIN LENGTH ON SENSITIVITY OF STX:

We prepared a few additional matrices which differ only in the surfactant component relative to the selected matrix (5% glycerol, 0.5% acetic acid, 0.1% PEG400, 0.5% PEG300, 0.025% sodium dodecyl sulfate, and water) to examine how surfactant chain length affects surface activity of STX. The surfactants are:

Hexanesulfonic acid sodium salt (0.025%).

Decanesulfonic acid sodium salt (0.025%).

Dodecanesulfonic acid sodium salt (0.025%).

Hexadecanesulfonic acid sodium salt (0.025%).

All but hexadecanesulfonic acid sodium salt showed some surface activity enhancement for SXT although the extent of

enhancement varied. As shown in Figure 18, the selected matrix (sodium dodecyl sulfate, trace D) in general showed better results and sensitivity. Hexanesulfonic acid sodium salt(trace A) and dodecanesulfonic (trace C) acid sodium salt yielded reproducible but much less sensitive signals. Hexanesulfonic acid sodium salt gave the lowest sensitivity. This means that a certain range of HLB (hydrophylic lipophilic balance) of surfactant should be secured depending on the polarity and size of the molecule to be analyzed. The sensitivity obtained by decanesulfonic acid sodium salt is as good as or better than that obtained by sodium dodecyl sulfate while the reproducibility is less.

On the other hand, hexadecanesulfonic acid sodium salt yielded totally unacceptable results. As shown in Figure 19, this large size surfactant failed to pull enough STX to the matrix surface ,and the sensitivity was further decreased as the long chain surfactant easily adsorbed on the silica surface and, in turn, strongly interacted with STX and yielded a very broad signal. In Figure 19, three replicate injections of 5ng STX yielded no recognizable peaks while an injection of 50ng STX yielded an extremely broad peak.

Coaxial Tee FAB/LC:

Jorgensen et. al. [34-36] described a coaxial tee as a method

of using capillary column (10-50 microns I.D.) for separation of peptides by introducing LC liquid phase and FAB matrix independently into the source. The two liquid phases were pumped independently and tended to increase resolution and sensitivity. The coaxial fused-silica capillary system consists of an open tubular fused silica (typically 10 um i.d. 150 um o.d.) inserted into a sheath column (typically 200 um i.d.by 350 um o.d.). When analysis of saxitoxin was attempted using this system, we could not obtain a significant increase in sensitivity over that obtained in the conventional direct probe analysis. This may be due to the fact that we used a matrix composed of 5% thioglycerol, 1% acetic acid and water while they used a matrix composed of 25% glycerol and water and that we used much faster flow rates for the analyte and the matrix (2 ul/min and 5 ul/min) than their flow rates (10-100nl/min and 0.5-1 ul/min). A syringe pump is required to deliver such a low flow rate for the analyte.

Detection of Neo-STX:

We examined the performance of the new matrix (5% glycerol, 0.5% acetic acid, 0.025% NaDOS, 0.1% PEG400, 0.5% PEG300) in detecting neo-STX by SIR. As shown in Figure 20, 50ng of neo-STX was reproducibly detected, while a fluctuation of sensitivity in detecting 5ng of neo-STX was observed. It should be noted that neo-STX did not give us the peak shape and

sensitivity as obtained with saxitoxin. However, we believe we can detect 500pg neo-STX with good reproducibility if the FAB conditions (including matrix) are optimized for neo-STX.

III. COOPERATIVE STUDY WITH OTHER GROUPS:

CZE (Capillary Zone Electrophoresis)/FAB

We visited the National Institute of Environmental Health Sciences (NIEHS) Mass Spectral Laboratory (laboratory of Dr. Ken Tomer) to learn about their CZE/FAB system constructed by Dr. Tomer and his assistants Lisa Deterding and Arthur Mosley [34-36]. The objective was to determine whether CZE will give us resolution and increased sensitivity for STX so that we could work in the picogram range with ease. As shown in Figure 21, the CZE system can easily detect 80pg of STX with excellent reproducibility and a signal to noise ratio of about 8:1. We believe it a fair statement that the CZE analysis could equal or better the 30 picograms found in ion spray by the Halifax group (Quillium et al.)[26]. Mosley and Deterding mentioned that the good sensitivity and reproducibility of their system are not due to the CZE but due to the design of their probe (coaxial design) and their matrix (25% glycerol and 75% water). The function of the CZE is separation or resolution. Mosley indicated that if we use an open tubular capillary instead of the CZE, the sensitivity will be either as good as that of the CZE or better.

The unique feature of their system is the very low flow rate of analyte (10-100nl/min.) and matrix (0.5-1ul/min.). They use syringe pumps to obtain such low flow rates.

Plasma Spray MS

Dr. Lawrence Hogge of the Canadian Research Council in Saskatoon, Saskt. examined the feasibility of using plasma spray MS for STX analysis. Plasma Spray ionization was not effective in detecting STX. Our rationalization is that STX exists as a charged form (salt) and it does not evaporate to give gaseous saxitoxin molecules by a thermal mechanism.

IV. DERIVATIZATION STUDY OF SAXITOXIN

No Mass spectra of STX by GC/MS has been reported. The difficulties in detecting saxitoxin by GC/Mass are due to the unique molecular structure of saxitoxin. Saxitoxin is unstable in an alkaline solution [5,9]. In neutral or acidic solutions, saxitoxin exists in a protonated form [5,9]. Therefore crystallized saxitoxin is in a salt form (acetate or hydrochloride). Saxitoxin (or salt) is very soluble in water, somewhat soluble in methanol, and insoluble in nonpolar organic solvents. Aqueous solutions cannot be injected into a GC column.

A methanol solution of STX can be injected, but no peak is ever detected by gas chromatography either in split/splitless mode or on-column injection mode. Molecules with a charge are not volatilized in the GC injection port and therefore saxitoxin molecules never get into the column.

We believe that it is desirable to derivatize saxitoxin before injection so that derivatized saxitoxin can be analyzed by GC/Mass. The usual GC-in-line derivatization technique is not applicable because derivatization of the guanidine group (charged site) requires an aggressive reaction condition or a long reaction time [37].

Our goal in derivative formation was to place a fluorescent or absorbing group on the guanidine imino nitrogen. ("a" positions in Figure 22) . After a number of attempts of derivatization we learned that these ring guanidine imino groups are chemically unreactive. Even imino groups of free guanidine molecule are resistant to chemical reactions [38]. In most of the attempts, STX was consumed and some products formed (monitored by TLC) but no meaningful mass was ever detected by GC/MS. We conclude that other amino groups ("b" and "c" positions in Figure 22) are more reactive than "a" imino groups; thus attack of a reagent occurs at "b" and "c" positions and "a" positions remain unreacted because of low reactivity and steric hindrance.

Analysis by TLC

In order to monitor the reaction product of saxitoxin, it was necessary to develop a system of chromatography to resolve the individual components. Two kinds of thin layer plates (normal phase Merck, 0.25mm and Whatman Inc. reverse C18 phase) were used. The solvent development system tried are shown in table 1.

Table 1. Resolution of saxitoxin by thin layer chromatography using various solvent systems

solvent system	ratio vol/vol	TLC plate	rf value
2% NH4OH in CHCl3:MeOH	4:1	normal	0.0
Ethanol:H2O:Ethyl acetate	10:4:2.5	normal	0.0
Acetonitrile:MeOH:H2O	1:1:1	reverse	0.0
Acetonitrile:MeOH:H2O	1:1:2	reverse	0.45
MeOH:H2O	3:1	reverse	0.0

The acetonitrile:methanol:water(1:1:2) solvent system gave excellent separation characteristics. Detection of STX on TLC Plates

Table 2. Reagents used to make saxitoxin visible on TLC plates.

Chemical reagent/1,2	Saxitoxin Response			
	Color/3		Fluorescence/4	
	22 C	140 C	22 C	140 C
Ninhydrin solution	no	no	no	no
20% methanolic sulfuric acid	no	aqua/black	no	yes
acidic p-anisaldehyde	no	pink/black	no	yes
hydrogen peroxide	no	no	no	yes

1/ Saxitoxin was spotted on reverse phase TLC plates and developed in acetonitrile: methanol: water (1:1:2)

2/ The chemical reagents were applied to the plates by spraying.

3/ Color was determined with the unaided eye.

4/ Fluorescence was determined by short and long ultra violet.

Saxitoxin turned aqua in color when treated with methanolic sulfuric acid and pink with acidic p-anisaldehyde. After heating for ten minutes on a hot plate, saxitoxin fluoresced. When heated to 140 C for 30 minutes after spraying with 20% H_2SO_4 or p- anisaldehyde, the color turned black.

(A). Acetylation.

A 10 ul solution of saxitoxin acetate (10 ug/ul), 30 ul acetic anhydride, and 100 ul pyridine are transferred into a 1 ml vial with a Teflon-lined cap. The vial is placed in an oven at 110-120oC for 48 hours. The content is dried under nitrogen at room temperature. The residue is dissolved in 100 ul methanol. A 1 ul aliquot is taken and injected for analysis. Some derivatized species of STX were detected but no saxitoxin was detected in the reaction product on a TLC plate. We were not able to detect any of the derivatized species by GC/MS.

(B). Dansyl Chloride

Twenty micrograms of STX was placed in a half dram vial to which 50 ul of dansyl chloride reagent was added and allowed to react for 1hr at 60 C. (The dansyl chloride reagent was prepared by dissolving 50mg of reagent in 1ml of acetone). The excess reagent was evaporated under nitrogen and the residue redissolved in acetone or methanol. Reaction products were studied by TLC, HPLC, and FABMS.

In another experiment, the reaction was run as described by Dunges et al. [39]. Briefly, 400 ul of ethyl acetate containing 500 ug of dansyl chloride and 5 to 10 ug of STX and solid K2CO3, was refluxed in a two-necked reaction vessel for 2 hours. Aliquots of the ethyl acetate were sampled and analyzed directly by FABMS. Alternatively, the ethyl acetate was evaporated to

dryness and the residue dissolved in acetone or methanol and the solution analyzed by TLC, HPLC [40,41], or FABMS.

Thin layer chromatography did not reveal any reaction products of STX. Elimination by HPLC coupled to a fluorescence detector was negative (no fluorescence product was formed); however, examination by ultra violet absorption at 254 nm revealed a reaction product at a retention time of 8,66. Unreacted STX was absent (Figure 25). We attempted to confirm the product by direct probe FABMS but could not find the expected mass spectrum.

The Dansyl reaction scheme that we expected for STX is described in Figure 23 i.e. sulfonyl bond on the imines on C8 and C2. However, we suspect that the reaction actually followed the scheme as in Figure 24 i.e bond at N9 or N7. It appears that the sensitivity of the reaction product in FAB is less than that of STX since we could not detect it. However, the product was detected by HPLC with a C18 reverse phase column as shown in figure 25.

(C). Edman Reaction

The chemistry involved in the Edman reaction (nitrophenylisothiocyanate or NPI) are described in Figure 26. The Edman reagent was prepared by adding 50mg of NPI to 1ml of

acetone. Fifty ul of 4-nitrophenyl isothiocyanate of this solution was placed in a solution containing STX (10 ug) and reacted for 1hr at 60 C. The reaction mixture was evaporated to dryness and the residue dissolved in acetone or methanol and analyzed by TLC, HPLC, and FABMS. A reaction product was detected on TLC but not on HPLC using both fluorescence and absorption detectors. No product was detected when analyzed by FABMS.

(D). p-Nitrobenzyl chloride.

Fifteen micrograms of STX was dissolved in acetonitrile and sodium hydroxide (20% aqueous) and then reacted with p-nitrobenzyl chloride for 6 hrs as described by Ross et al. [42]. The acetonitrile solution was evaporated to dryness and the residue dissolved in acetone and analyzed by FABMS. No reaction products of p-nitrobenzyl chloride and STX was detected by FABMS.

(E). Reduction by LIALH4

Dried STX 10ug and 10mg LiAlH4, and THF 4ml were added to a vial. The mixture was stirred at room temperature for 3hours. The reaction product was analyzed by direct probe EI MS. The mass of reduced STX was not observed. Afterwards, the major component of the reaction product was separated by TLC and

extracted with methanol. The methanol solution was analyzed by GC/MS. No peak was observed. The purpose of this reaction was to convert the guanidine imino groups to primary amino groups to facilitate further derivatization as shown in Figure 27. We suspect that the reaction condition of LIALH4 reduction is strongly alkaline and caused decomposition of STX.

(F). Addition of a Alkyl Chain Using Hexanoyl Chloride.

This reaction was tried to increase surface activity by attaching a long alkyl chain on one of the ring hydroxyl groups (C12) of STX. In a 2ml Reacti-Vial, 10 ul SXT solution (1 ug/ul) was added and dried under nitrogen. Fifty 50 ul hexanoyl chloride was added and the vial was sealed using a Teflon-lined septum and a screw cap. A disposable syringe needle was allowed to go through the septum to allow escape of HCl. The Mixture was heated at 130-150 C for 4 hours. After reaction the mixture was heat-dried under nitrogen and the residue was dissolved in methanol and analyzed by both GC/MS and direct probe FAB. No peak with a reasonable mass was observed by either method.

(G). Reaction with Propylene Oxide and TFAA

Bjorkhem et. al. [43] reported derivatization of creatine using propylene oxide and TFAA. Creatine has a cyclic guanidine group like saxitoxin. We tried this method for saxitoxin. The

expected reaction scheme is described in Figure 28. To 10 ug dried STX, 100 ul methanol and 100 ul propylene oxide were added. The mixture was heated at 70 C for 30 min. and evaporated to dryness under nitrogen. One hundred ul TFAA and 200 ul ethyl acetate were added and the solution was heated at 37 C for 30min and evaporated. The residue was dissolved in 50 ul methanol and analyzed by GC/MS. The mass spectra of some of the chromatographic peaks are shown in Figure 29. Unfortunately, none of them matched the expected reaction products. All of the chromatographic peaks of the reaction products were also observed from a blank product, prepared from propylene oxide, TFAA, and ethyl acetate, without addition of STX.

(H). Silylation.

The following silylating reagents were tested for their reactivity with STX: TMCS (trimethylchlorosilane), MSTFA (N-methyl- bis (trifluoroacetamide)), BT (mixture of N,O-bis (trimethylsilyl) acetamide(BSA) and TMCS), TBT (mixture of trimethylsilylimidazole (TMSI), N,O- bis (trimethylsilyl) acetamide and TMCS), and TMSDEA (N-trimethylsilyldiethylamine). The silylating reagents are shown in Figure 30. None of the silylating reagents showed the expected products when analyzed by MS.

(I). Methylation followed by silylation

Dried saxitoxin was first treated with Methelute, dried and treated with TMCS or MSTFA with the intention of activating reactivity of the guanidine imino groups to the silylating reagents by methylating the adjacent amino groups. None of the above reactions gave the expected products when analyzed by both GC/MS and FABMS.

(J). Methylation.

1. Methelute (trimethylammonium hydroxide)

When aqueous saxitoxin was reacted with Methelute at 120C, a product was formed with a m/z value of 254. When it was reacted at 150 C, the product had a major peak of m/z 396. It was calculated that the heptamethylated saxitoxin should have a molecular ion minus one (396), hence we were excited at the possibility of detecting low picogram amounts of the toxin. See Figure 31 for the analysis of the reaction product using SIR and selection of m/z 396 as the determinant. Full scale detection was obtained on what we thought was 200 picograms of permethylated STX. We falsely rationalized reaction schemes for the products of m/z 254 and 396 as in Figure 32 and 33.

Experiments were conducted to verify the authenticity of the m/z 396 fragment as being due to the reaction product of STX and Methelute. When saxitoxin and Methelute were placed in a

sealed glass capillary and heated at 150C for 2 hours, formation of the product having the m/z 396 was minimal. When Methelute alone was heated at high temperature and taken to dryness, the fragment with m/z 396 was formed thus proving that the reaction product is not STX related. We have not accounted for the structure of the fragment containing m/z 254 and 396 but it does not appear to be related to the supposed methylated STX species.

2. METHYLATION BY CH3I, NaOH IN DMSO

Based on well-known procedure of permethylation of carbohydrates [44,45], ten micrograms of dried SXT, 100 ul DMSO, 50 ul CH3I and a pellet of NaOH were added in a 2ml Reacti-Therm vial. The mixture was stirred for 30 min. at room temperature and 500 ul water was added to the mixture. After neutralization with dilute HCl to pH 7, the mixture was extracted by CHCl3. GC/MS analysis showed a very broad peak whose mass values are all less than 300. The permethylated product was not detected or alternatively , was not formed.

3. METHYLATION BY CH2N2

Thirty three milligrams of N-methyl-N-nitroso-N'-nitroguanidine and 125 ul water were added to the inner tube of the diazomethane generator. Ten ug dried SXT and 750 ul THF were added to the outer tube. The diazomethane generator unit was

assembled and 150 ul 5N NaOH was slowly added to the inner tube by a syringe. The bottom of the outer tube was cooled on an ice bath. After 60 min., the THF solution in the outer tube was dried under nitrogen, dissolved and analyzed by GC/MS. No meaningful peak was observed upon analysis by both HPLC and CFFAB.

4. METHYLATION BY MAGIC METHYL

Ten ug STX, 100 ul methylene chloride, and 5mg Magic Methyl (trimethyloxonium tetrafluoroborate) were added to a 1ml Reacti-Vial. The mixture was stirred for 3 hours at room temperature. After reaction, the reaction product was analyzed by direct probe FAB using thioglycerol as the matrix. No peak over mass 300 was observed. Next 200 ul water was added to the mixture and the mixture was neutralized by sodium carbonate to pH 7. The solution was extracted by 1ml chloroform. The Chloroform extract was analyzed by GC/MS. No meaningful peak was observed.

5. Methyl-8 (Dimethoxy-(N,N-dimethylamino)-methane).

1 ug of dried saxitoxin (under N2, room temperature) and 20 ul Methyl-8 were added in a glass capillary. The capillary was flame-sealed and placed in a oven at 150c for 2 hours. No product peak was observed by GC/MS.

V. Study of Purification and LC Separation of STX

We believe that in order to detect saxitoxin in a biological sample, the sample must be first chromatographed, to separate a saxitoxin-enriched fraction, before it is applied to a mass spectrometer. We briefly tested if saxitoxin can be separated using commercial minicolumns. The tested columns are Bandelute RP C-18 (octadecyl) and CBA (carboxylic acid) columns. Saxitoxin is strongly retained in both columns. Both columns have a considerable amount of residual silanol groups and the silanol groups firmly capture saxitoxin. In order to release trapped saxitoxin addition of silanol group blocking reagent such as triethylamine is required. Thus 3 ml triethylamine solution (10 mM) buffered with acetic acid to pH 7 is needed to pass 10 ul saxitoxin solution (10 ug/ ul) through a 0.5 ml CBA column. Saxitoxin can be separated by first being trapped in a column and second being released with a silanol group blocking reagent. Separation procedure must be adjusted and modified depending upon the property and composition of a biological sample.

In a personal communication with Dr. Harry Hines, we learned that U.S. Army Medical Research group developed an excellent purification process for STX (recovery >90%) by injecting a raw biological sample onto a CBA column followed by elution with pH 4 acetic acid solution. This information is very helpful as a

guide for elution studies.

Bonded Column and Packed Column

C-8 Bonded Column (50u, 5m, Lee Scientific)

We ordered a special custom-designed bonded capillary column for resolution of STX prior to analysis by CFFAB. Prior to our inquiry, there had been no commercially available bonded column that had an i.d.of 50 microns with a ligand chain length of 8 or more. The performance of this column for STX, however, was not satisfactory. Using the selected matrix (5% glycerol, 0.5% acetic acid, 0.1% PEG400, 0.5% PEG300, 0.025% sodium dodecyl sulfate, and water), we were only able to obtain broad and weak signals as shown in Figure 34. The wide band width may be due to adsorption of glycerol in the bonded phase and consequent sticky retention of STX in the glycerol impregnated bonded phase or due to adsorption of the surfactant anion in the bonded phase and consequent retention of positively charged STX by the anionic attraction. For FAB analysis of STX, glycerol and a surfactant are inevitable, thus a solution to the band broadening should be pursued by minimizing the adsorption effect.

Packed Capillary Column

Sullivan et. al. [18,19] reported a reversed phase HPLC determination of PSP toxins using a polystyrene divinylbenzene resin column (Hamilton, PRP-1). They were able to successfully separate PSP toxins and detect 100 pg STX by fluorescence following post-column alkaline periodate oxidation. We prepared our own PRP-1 column by packing the Hamilton PRP-1 stationary phase in a silica capillary (20cm, 530 uI.D.) to couple this HPLC technique to FABMS. Such micro LC technique has been developed to meet requirements of micro-analysis [46,47] and is appropriate for interfacing with MS. Sullivan and coworkers used a gradient eluent system as follows: 100% A to 100% B in 20 minutes.

A: Water with 1 mM C6 and C7 sulfonic acid (NH4 salt) and 1.5 mM ammonium phosphate (pH 7.0).
B: 50% methanol and 50% water with 0.5mM C6 and C7 sulfonic acid (NH4 salt) and 5.0 mM ammonium phosphate (pH 7.0).

STX eluted after 20 min., thus solvent B is effective in eluting STX from the column described above. However, both solvent A and B are not appropriate matrices for FAB analysis. Thus we prepared three new eluents as follows:

(1). The selected matrix.

(2). 5% Thioglycerol, 1% TFA and water.

(3). 50% selected matrix + 50% solvent B

Unfortunately we were not able to obtain a recognizable signal for 500ng STX with all of these matrices. The result of matrix (2) is shown in Figure 35, as an example. This failure may be due to the different retention time owing to the change of eluting solvent. We believe well-controlled (pH 7.0) ion pairing mechanism is the dominant process in the original HPLC method while retentive adsorption of STX in the polar sites of the impregnated stationary phase occurs in our experiment.

A gentle FAB matrix, in essence, cannot be used as a HPLC eluent. This dilemma can be solved if a coaxial design of Jorgenson et. al. [3,4] is adopted. That is, the HPLC eluent is mixed with the FAB matrix post column. The flow rate of HPLC eluent should be much less than that of the FAB matrix, and this requires a syringe pump. Alternately a CZE (capillary zone electrophoresis) system can be coupled to FABMS without use of a syringe pump. These possibilities may be carefully considered in later studies.

VI. Summary and Future Plans

We have developed an improved CFFAB technique where 500 pg STX can be reproducibly detected using an open bore

silica capillary column (25 micron i.d.) and carefully chosen matrices. This partially satisfied the requirement of detection of saxitoxin in the midpicogram range.

We have tried various derivatizations of STX expecting some products of improved sensitivity for either GC/MS or FABMS only to have little fortune. The guanidine groups in the STX rings are resistent to chemical reactions.

We will keep improving CFFAB technique. We will do our best to obtain the frit tip from VG Instruments, LTD. We will further optimize the matrix exploring effects of surfactant concentration, etc. We will also obtain a 60nl injector to improve sensitivity by narrowing peak width.

We will further study methods of coupling of a column to FAB/MS and of purification of raw STX samples.

We will examine how DCI works for STX.

We will attempt cooperative work with other groups, for example, analysis of STX by Sciex Ion Spray Mass Spectrometry.

We will test a HPLC/ electrochemical detector system as a supplementary technique of STX identification.

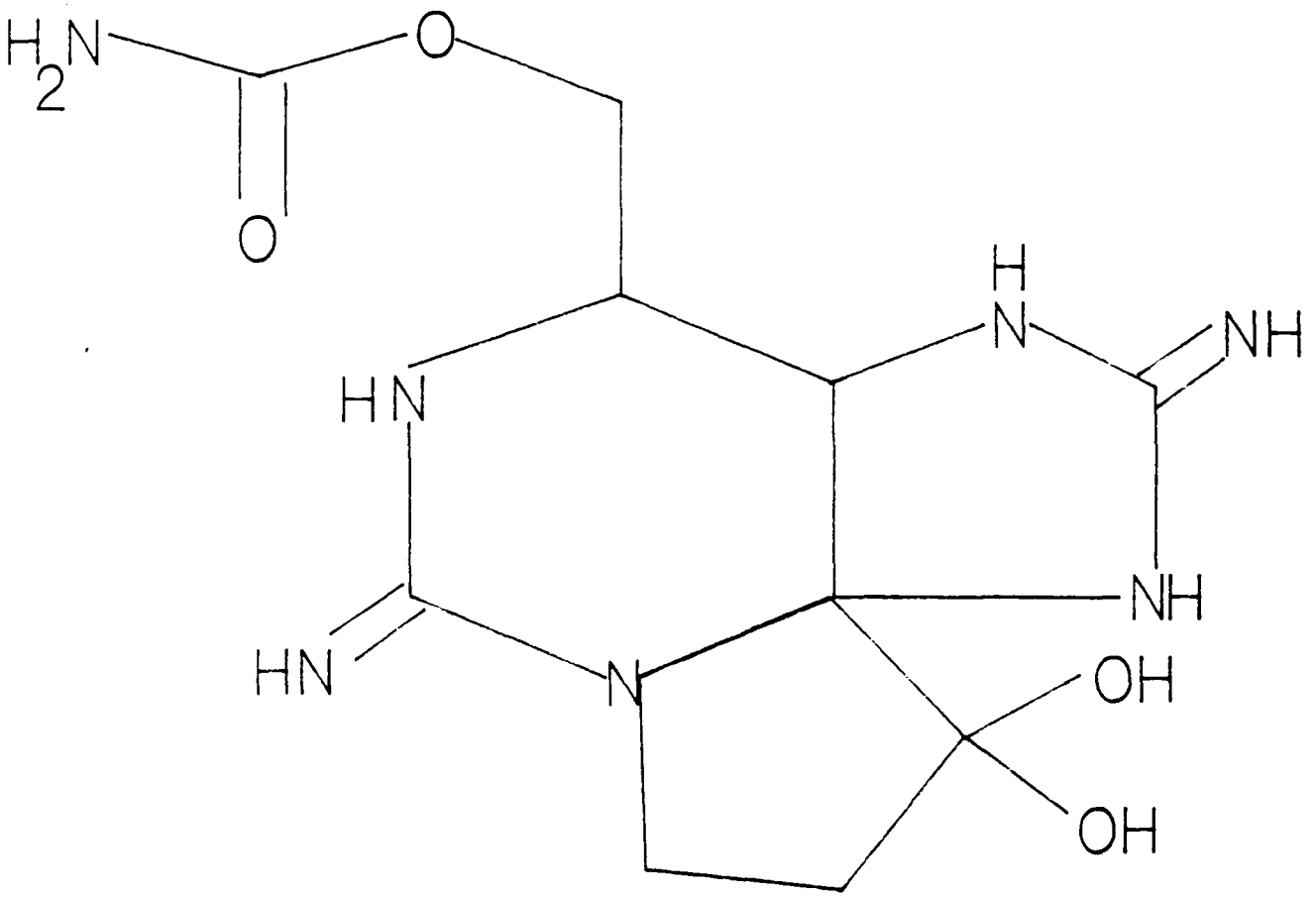


Figure 1. The structure of saxitoxin.

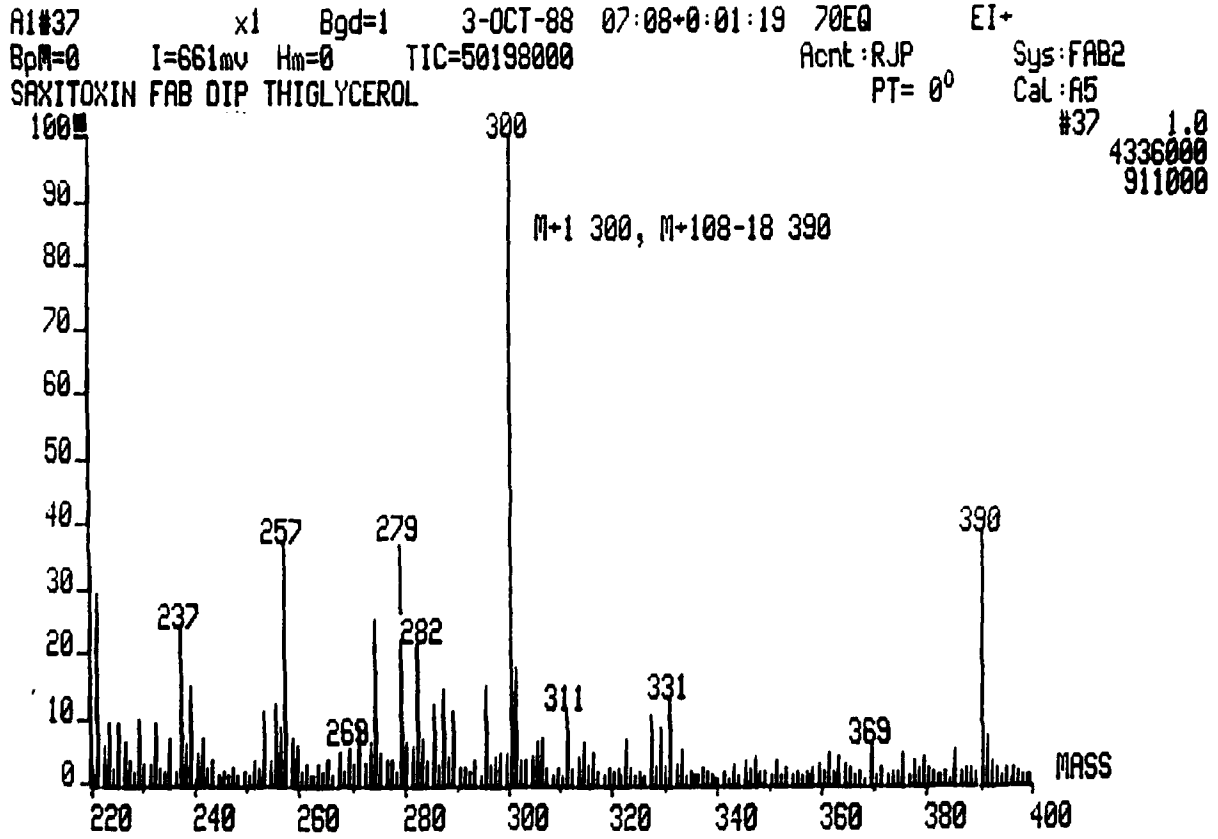


Figure 2. Fast atom bombardment mass spectrum of saxitoxin in thioglycerol:water (95:5) using CFFAB. The base peak recorded was 300 (molecular ion) and m/z 282 (molecular ion minus water).

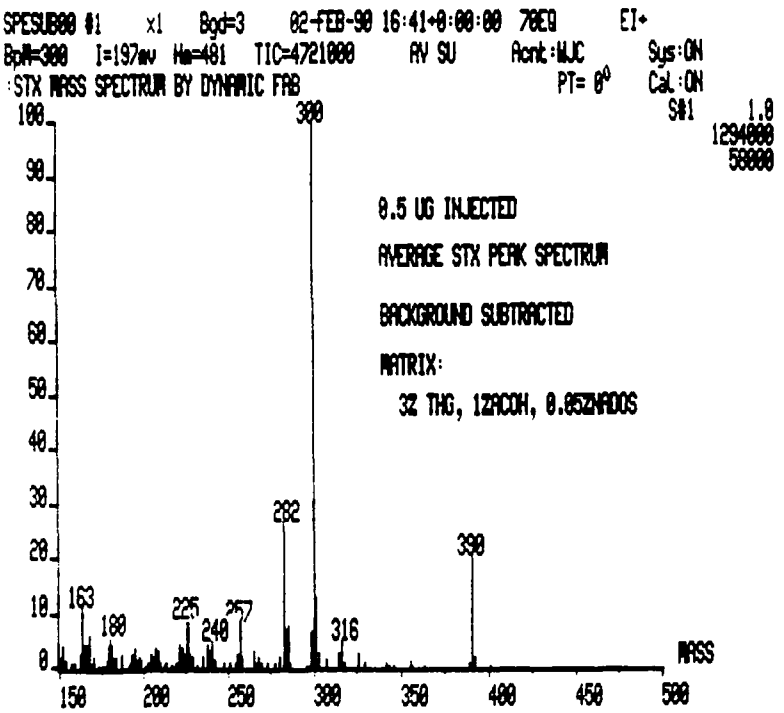


Figure 3. The background subtracted Mass spectrum of STX by CFFAB. The matrix composition is 3% thioglycerol, 1% acetic acid, 0.05% sodium dodecyl sulfate, and water. Half microgram was injected.

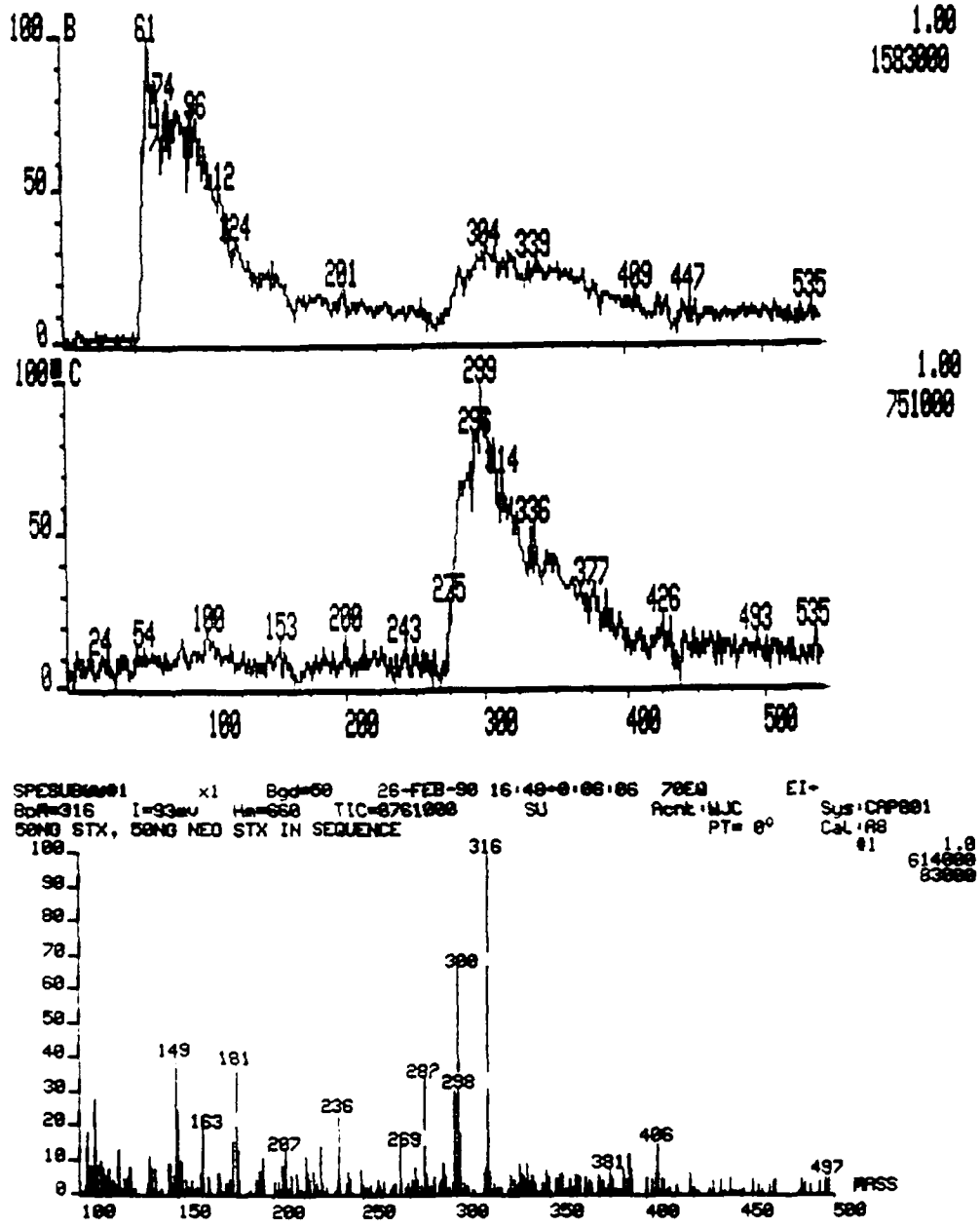


Figure 4. Detection of SXT and Neo-SXT and the background subtracted FAB mass spectrum of neo-SXT. Trace B is the chromatogram of 50ng of SXT traced at its molecular ion (MH+=300). Trace C is the chromatogram of 50ng of neo-SXT traced at its molecular ion (MH+=316).

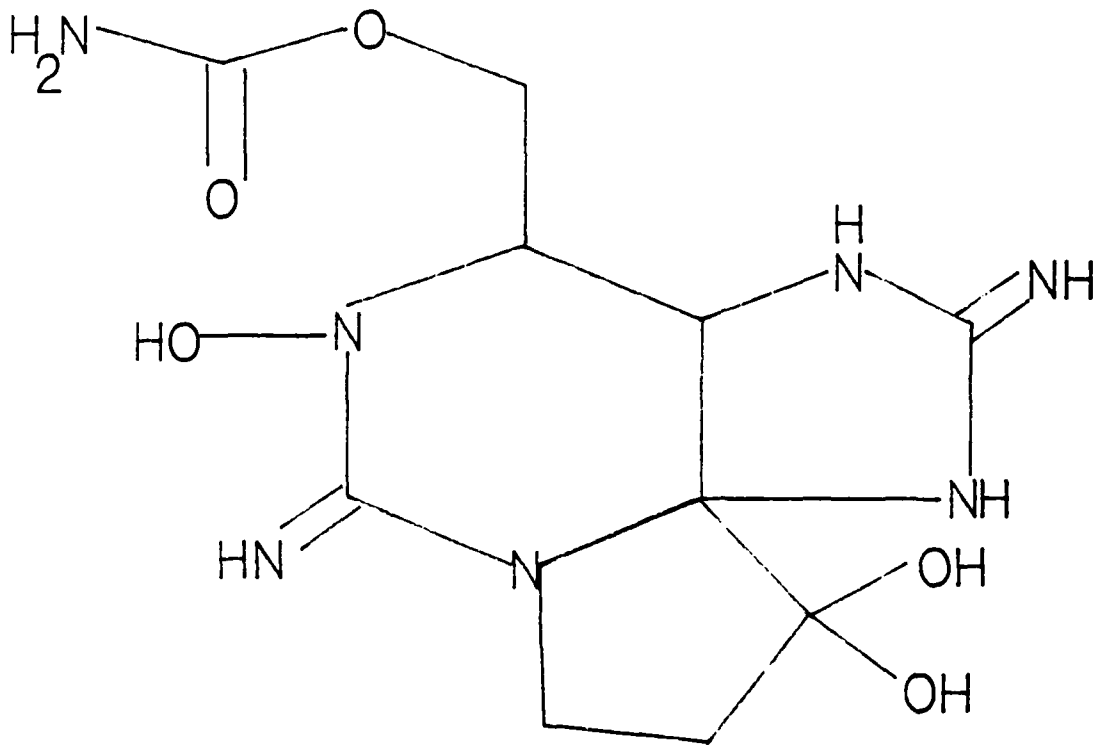


Figure 5. The structure of Neo-Saxitoxin.

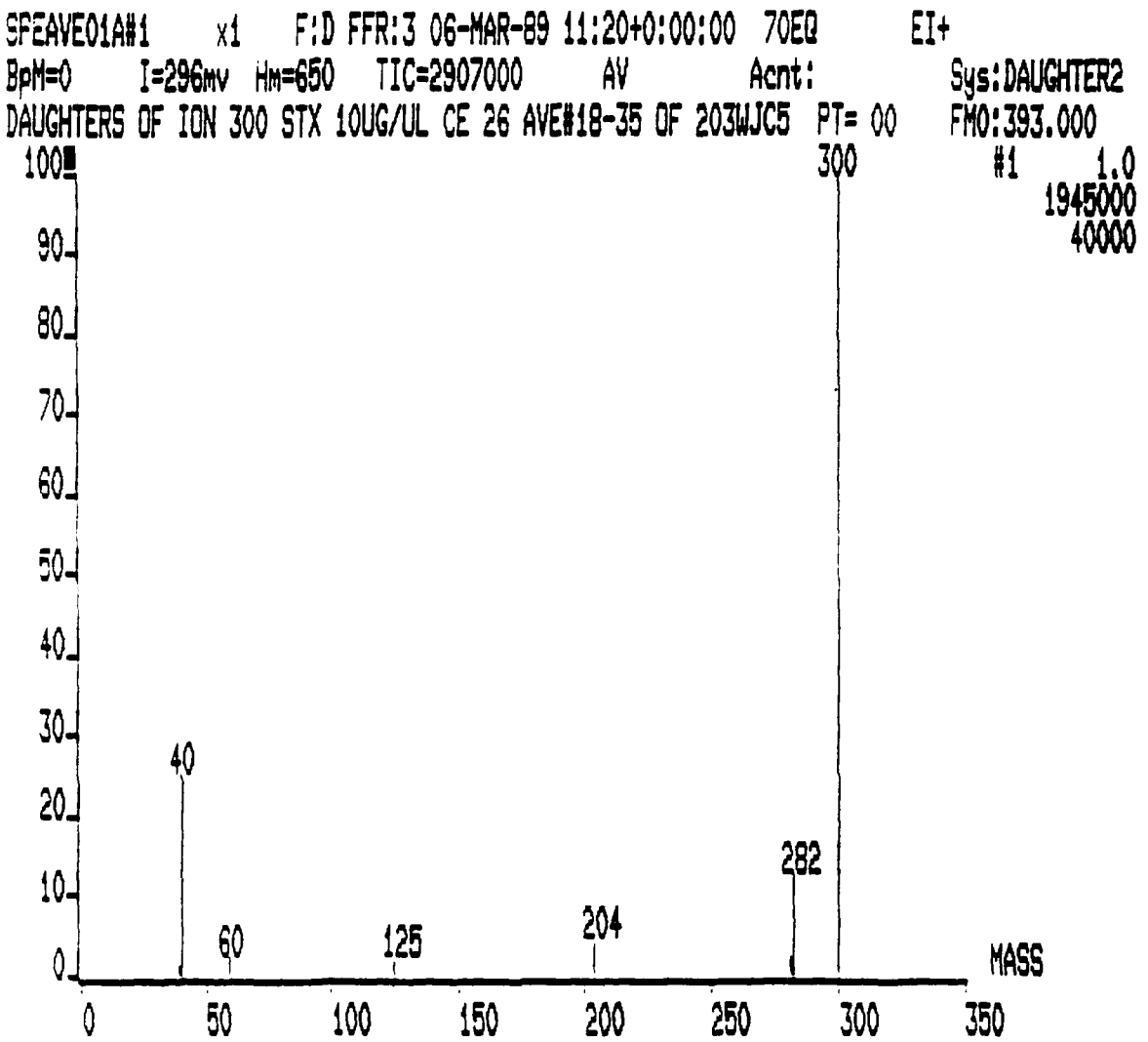
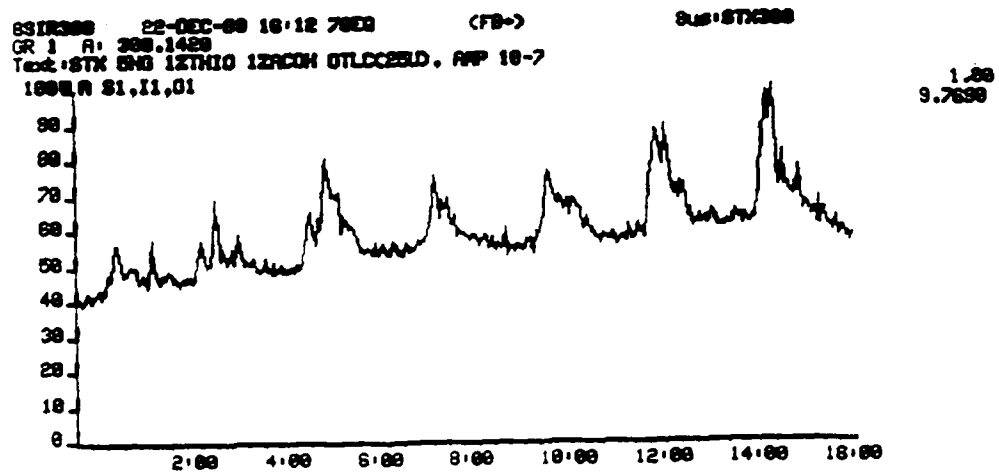


Figure 6. The MS/MS spectrum of daughters of STX molecular ion (MH+=300). Collision gas ;5X10-5 torr Ar. Collision energy;26eV.

(A)



(B)

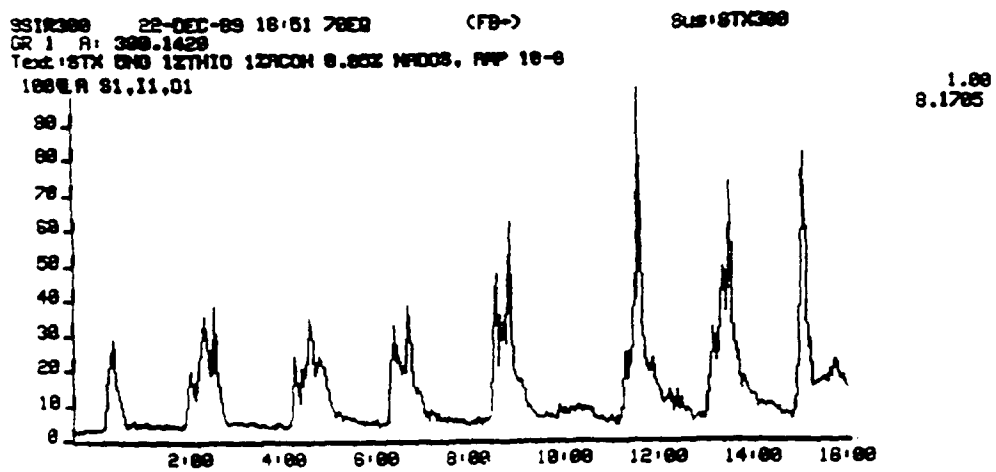
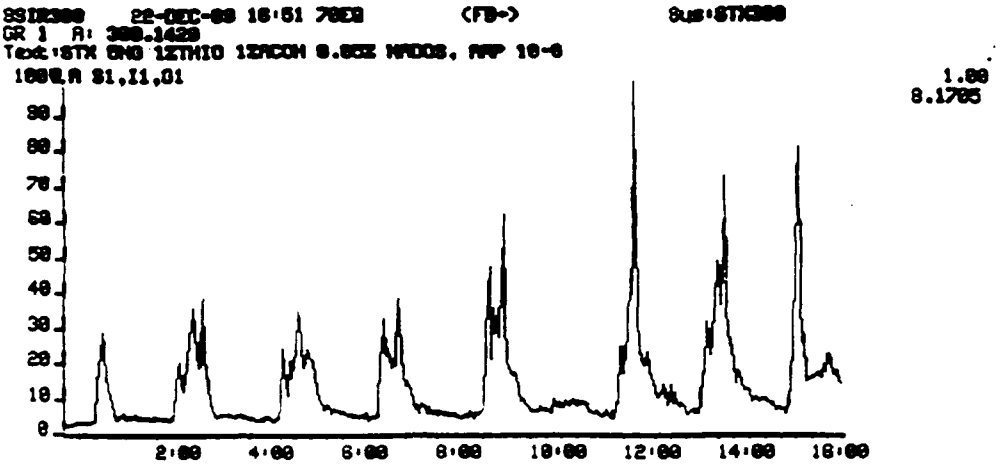


Figure 7 . Effect of surfactant (0.05% sodium dodecyl sulfate) on the detection of saxitoxin (STX) in Continuous Flow FAB (CFFAB) using an open tubular (25 i.d. micron) capillary loop injection method. (A) Multiple injections of 5 ng of STX without the addition of SDS. Note the lack of resolution indicating that the STX sample is not on the surface of the matrix where it can be ionized by the xenon beam. (B) Multiple injections of STX using SDS in the matrix. Note that the resolution has improved, the peak width is about 1.5 minutes and the base line is near 5 percent.

(A)



(B)

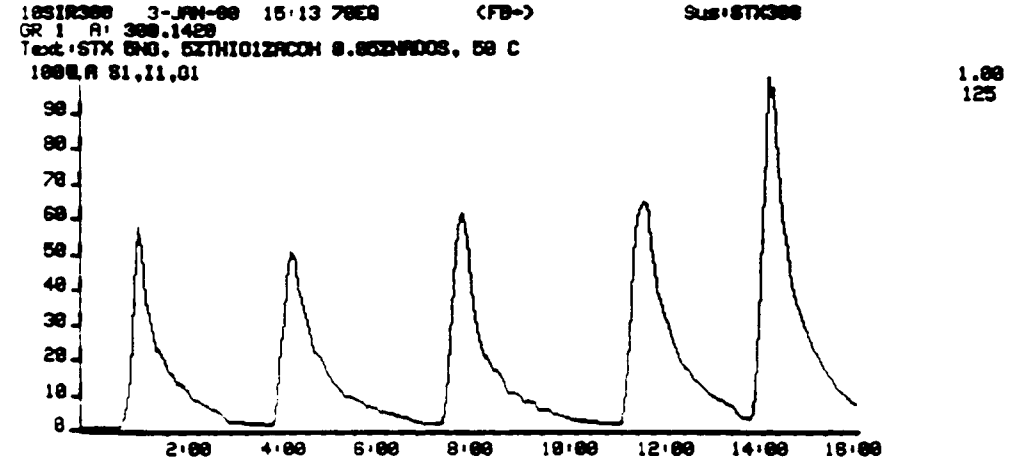


Figure 8 . Effect of the concentration of thioglycerol in the FAB matrix on sensitivity and chromatographic peak shape of STX in the CFFAB mode when injected by the open capillary tube method.(A). Multiple injections of 5 ng of STX using a 1% thioglycerol (THG) and 0.05 % SDS and 1% acetic acid. (B). Same injection as in A but using 5% THG in the matrix. Note the improvement in peak shape, resolution and reproducability..

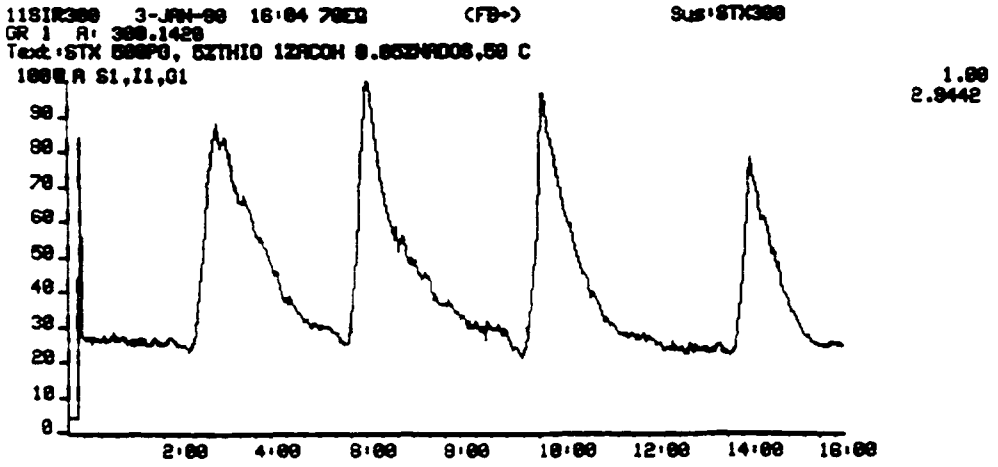


Figure 9 . Detection of 500 picograms of STX by CFFAB using as a matrix, 5% THG, 1% acetic acid and 0.05% SDS. Note the peak size and geometry, relatively narrow peak width (~1.5 minute) and reproducability of multiple injections.

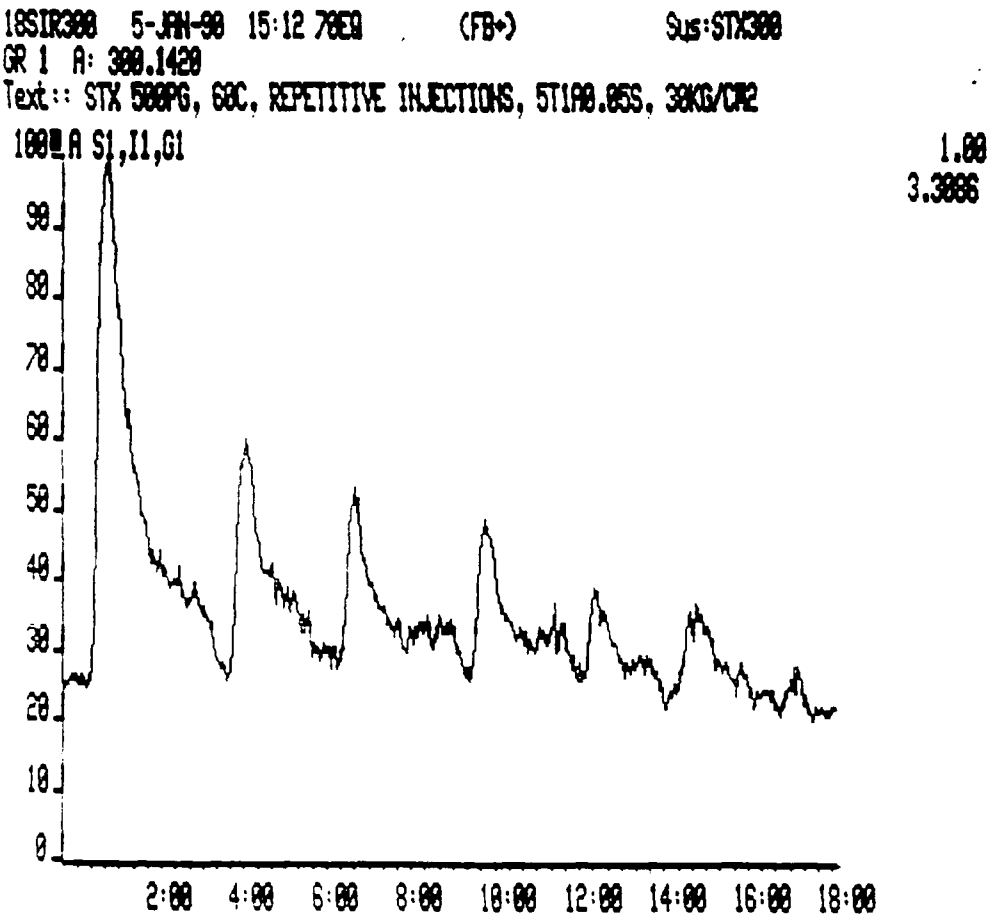


Figure 10. Multiple (repetitive) injection of 500 picograms of STX using 5% THG, 1% acetic acid and 0.05% SDS and a open tubular capillary (25 microns i.d.) injection method in CFFAB. Although each injection was detected by the analyzer, the peak shape and sensitivity was affected and was not linear. This may be due to contamination of the probe tip with THG or some phenomenon that we do not understand. This is an example of the problem encountered at low concentrations. Although STX is detectable at 500 picograms, its quantitation would have to be done with an internal standard i.e deuterated STX.

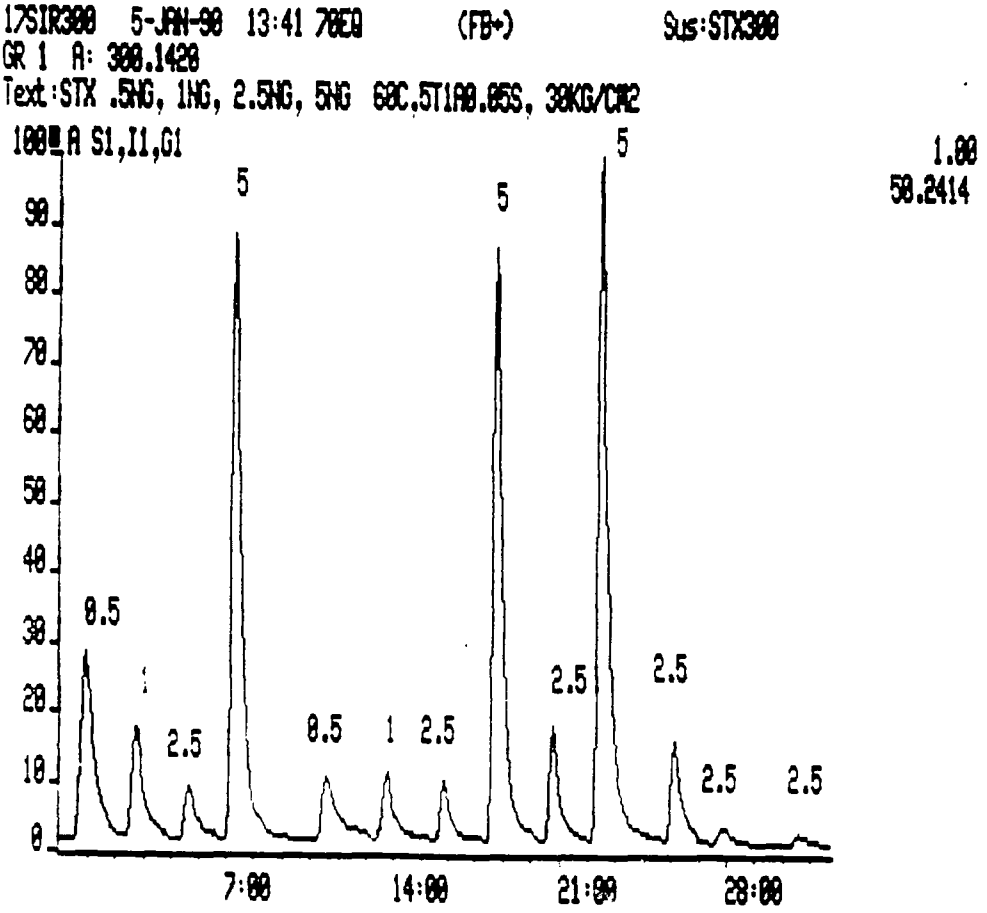


Figure 11 . Multiple injections (3) of STX (500 pg, 1 ng, 2.5, ng and 5 ng) by CFFAB using capillary (25 micron i.d.) loop injection and a matrix made up of 5% THG, 1% acetic acid and 0.05% SDS. The probe temperature was estimated as 60 C and the flow rate about 4 to 5 ul/ min. Note the improvement in peak shape and size particularly at 5ng STX. Although 500 pg, 1ng and 2,5 ng injections are detected, the response is not linear and reproducability is poor. We are working on improving the probe tip design i.e. fritted probe tip being developed by VG.

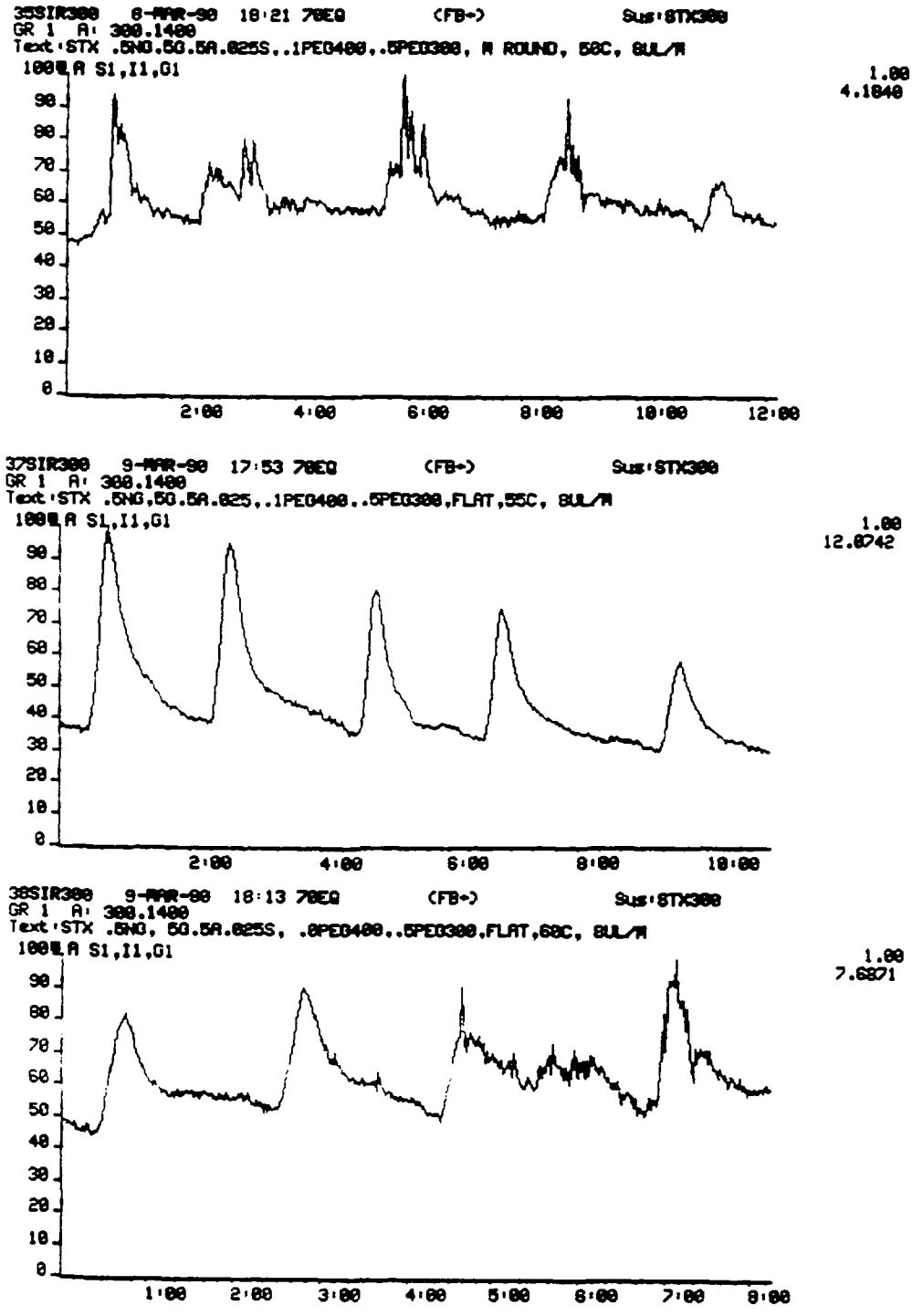


Figure 12. Effect of tip temperature. (A) Multiple injections of 500 pg of SXT using the selected matrix. Flow rate;8 ul/min; tip temperature;50C. (B) Multiple injections as in A but at 55C. (C) Multiple injections as in A but at 60C.

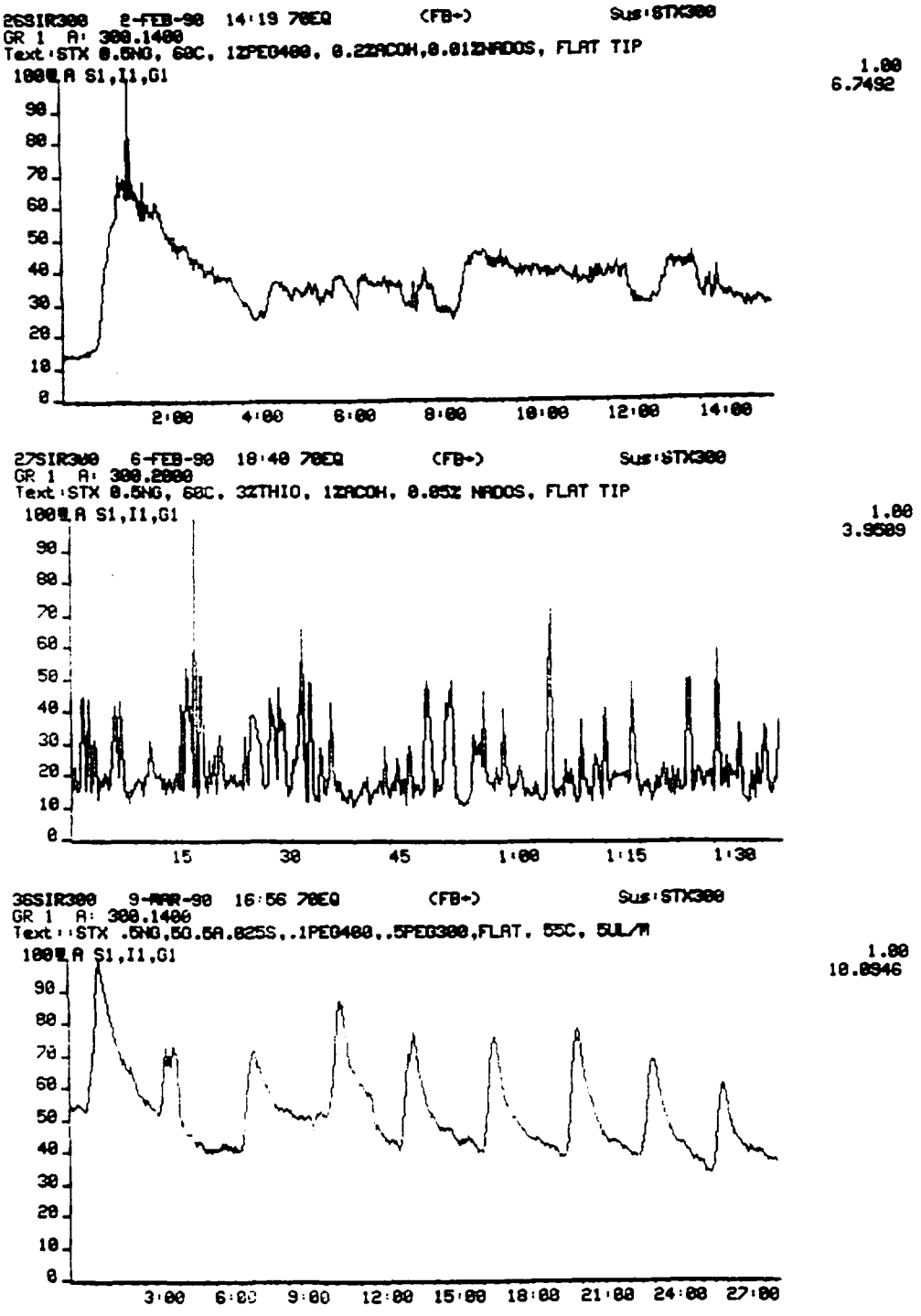


Figure 13. Comparison of matrices (matrix effect) in multiple injections of 500pg of SXT. (A) Matrix; 1% PEG400, 0.2% acetic acid, 0.01% NaDOS. Flow rate;4 ul/min. Tip temperature;60C. (B) Matrix; 3% thioglycerol, 1% acetic acid, 0.05% NaDOS. Flow rate;4 ul/min. Tip temperature;60C. (C) Matrix; 5% glycerol, 0.5% acetic acid, 0.025% NaDOS, 0.1% PEG400, 0.5% PEG300, flow rate 4 ul/ min.; tip temperature 60 C.

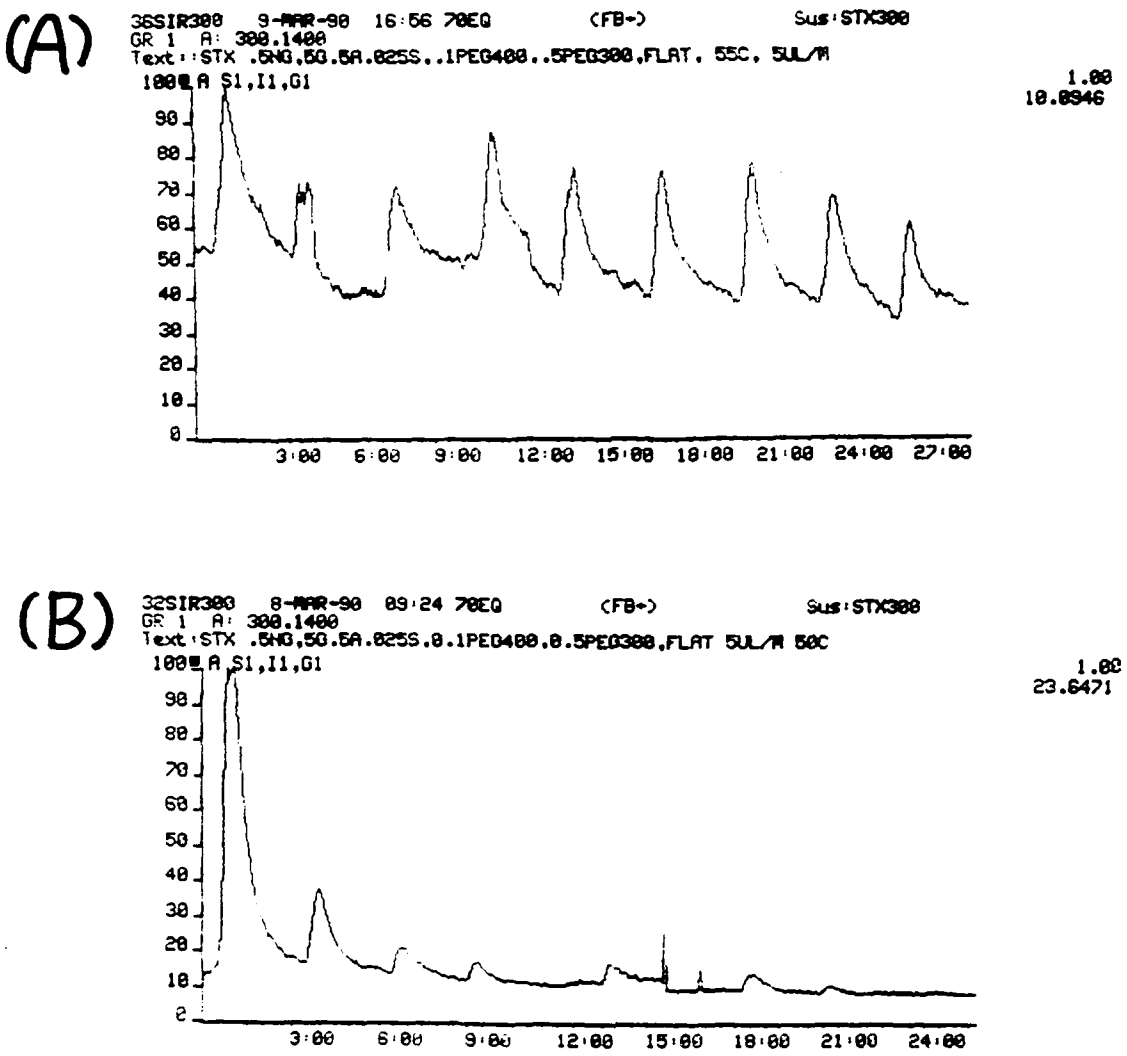


Figure14 . Effect of conditioning (i.e. cleaning) the tip. (A) Multiple injections of 500pg of SXT after conditioning (See the text for details of conditioning) using the selected matrix (5% glycerol, 0.5% acetic acid, 0.025% NaDOS, 0.1% PEG400, 0.5% PEG300). Flow rate;5 ul/min. Tip temperature; 55C. (B) Multiple injections as in A but without conditioning. Flow rate;5ul/min. Tip temperature; 50C.

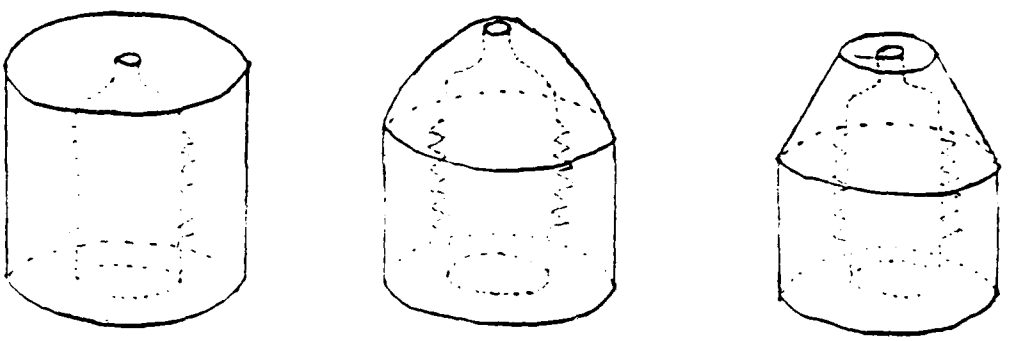


Figure 15. Design of probe tips used in these experiments.

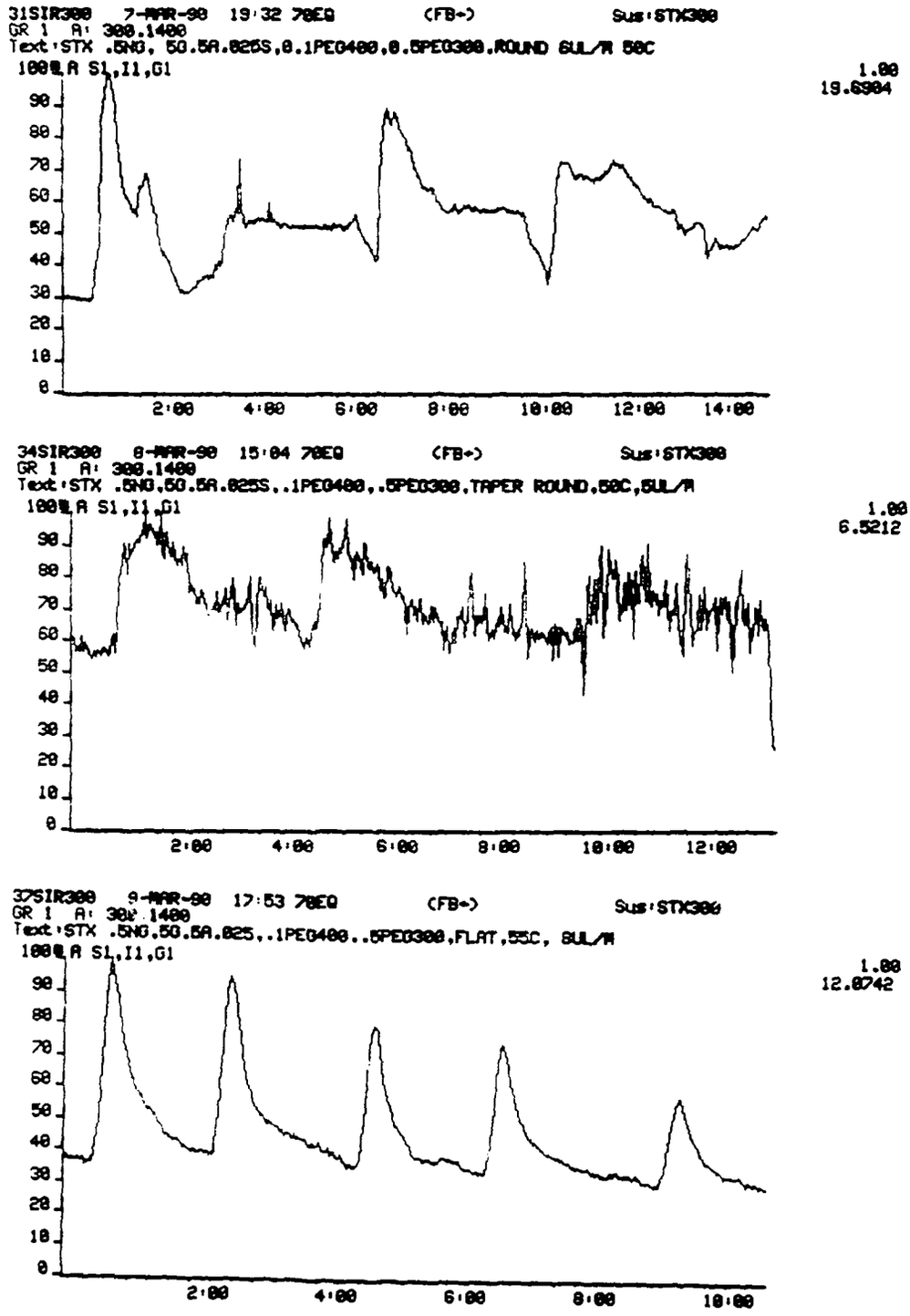


Figure 16 . Effect of tip geometry on detection and sensitivity of saxitoxin. (A) Multiple injections of 500pg of SXT using the selected matrix and the round tip at 50C. (B) Multiple injections as in A but using the tapered tip at 50C. (C) Multiple injections as in A but using the flat tip at 55C.

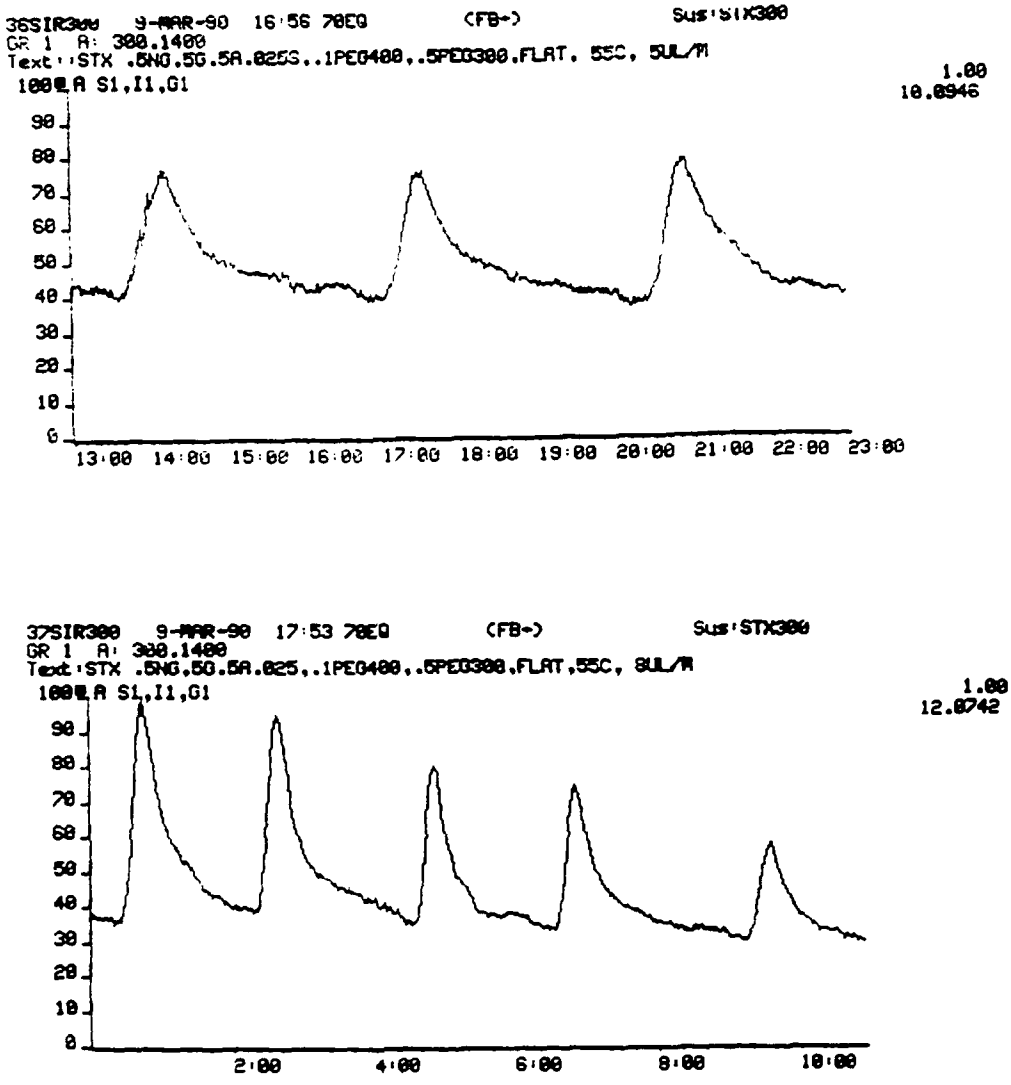


Figure 17. Effect of matrix flow rate on peak width. (A) Multiple injections of 500pg of SXt using the selected matrix with flow rate=5 ul/min. (B) Multiple injections as in A but with flow rate=8 ul/min. Peak widths in B are narrower than those in A.

Figure 18. Surfactant Effect on Selectivity and Reproducibility in SIR Analysis of STX at M=300.

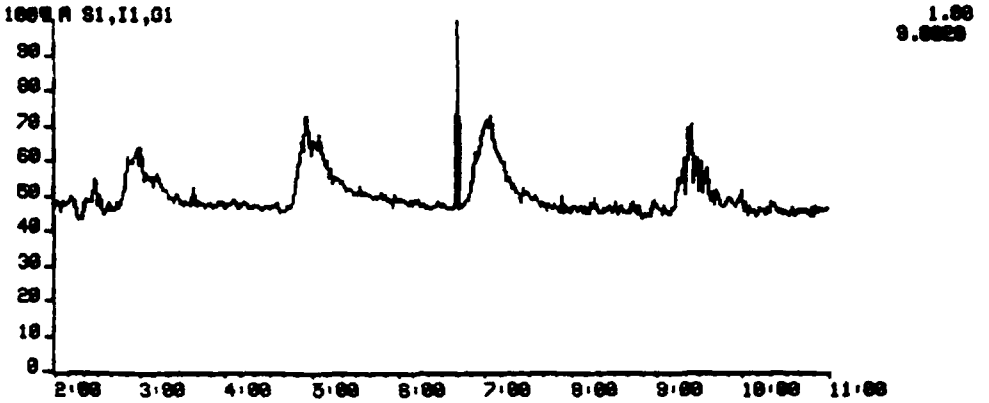
(A). Multiple injections of 5ng STX using a matrix composed of 5% glycerol, 0.5% acetic acid, 0.1% PEG400, 0.5% PEG300, 0.025% hexanesulfonic acid sodium salt, and water.

(B). Multiple injections of 5ng STX as in A but using 0.025% decanesulfonic acid sodium salt.

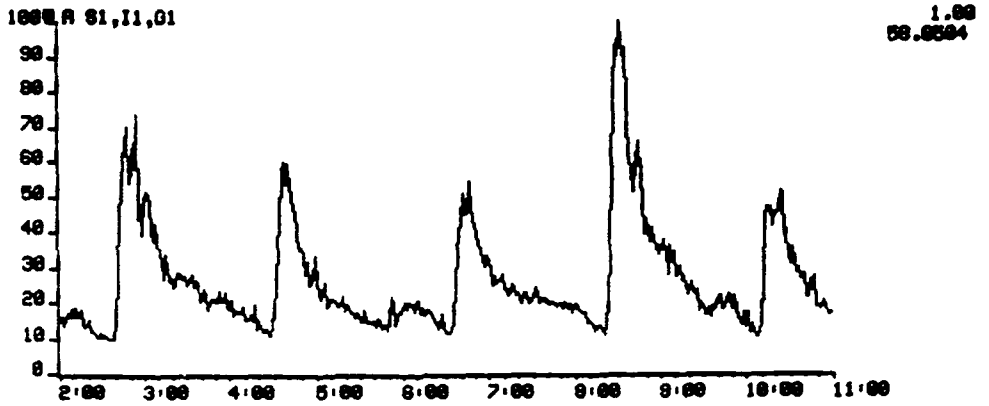
(C). Multiple injections of 5ng STX as in A but using 0.025% dodecanesulfonic acid sodium salt.

(D). Multiple injections of 5ng STX as in A but using 0.025% sodium dodecyl sulfate (the selected matrix).

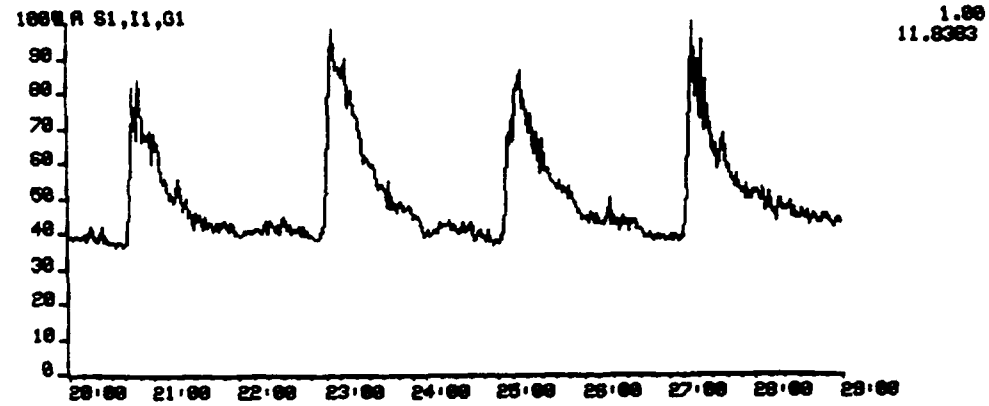
A



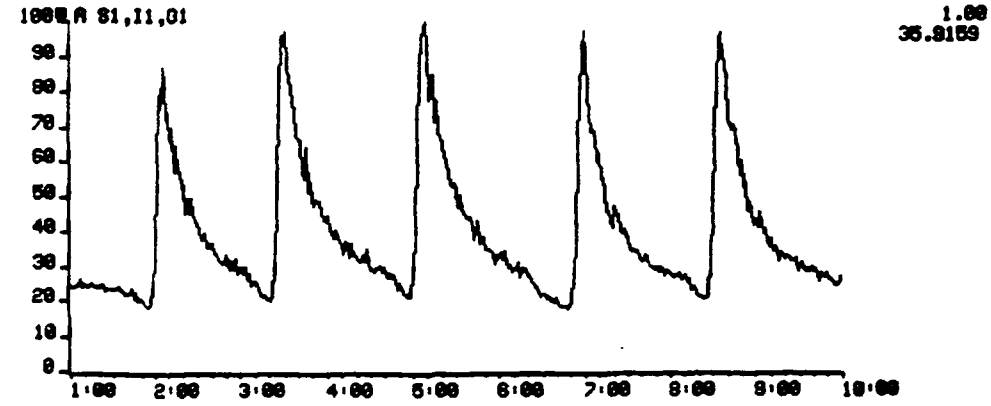
B



C



D



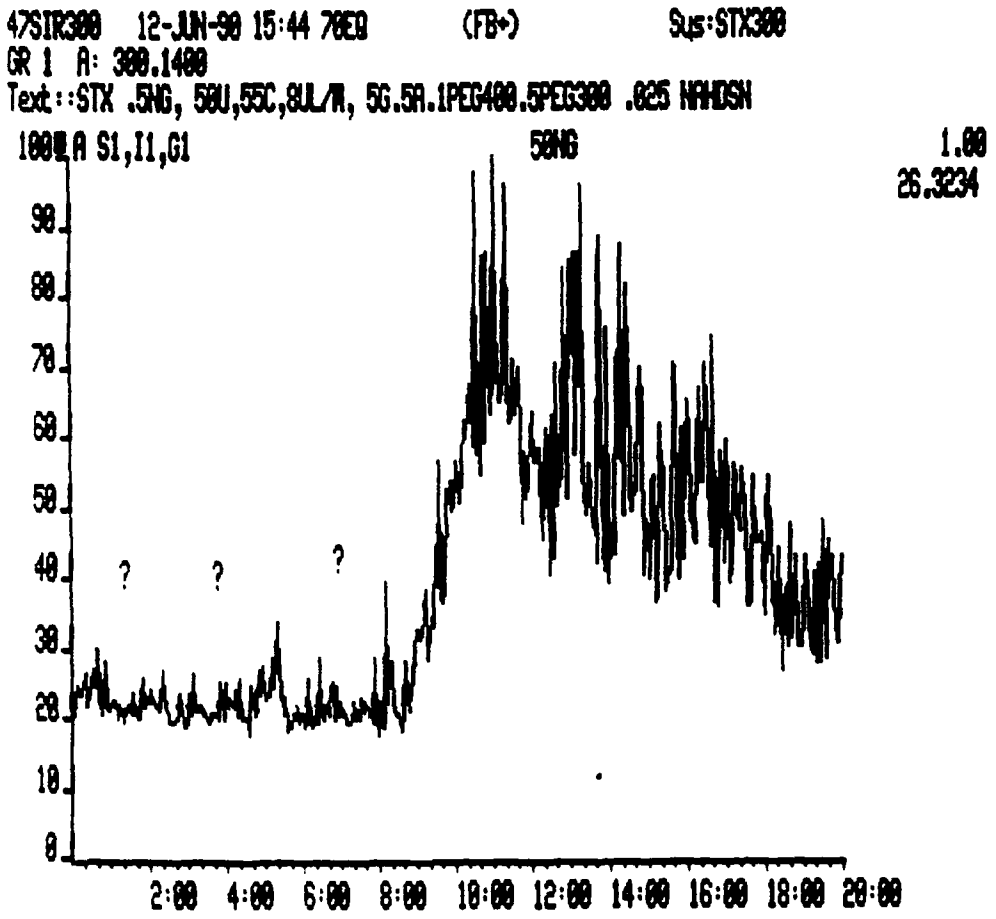


Figure 19. The Effect of a long chain surfactant on sensitivity and peak width. Three injections of 5ng STX gave no recognizable signals while a injection of 50ng STX yielded a very broad peak.

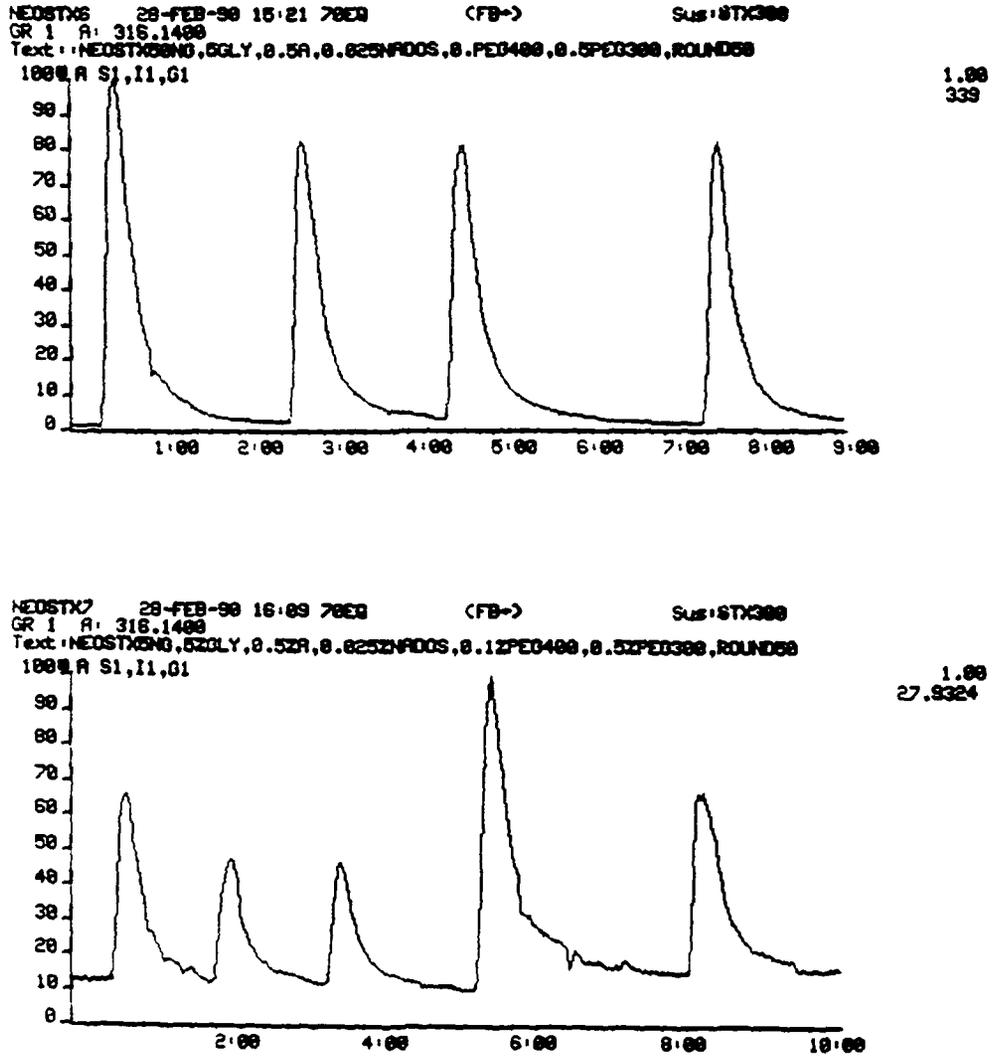


Figure 20 . Detection of Neo-STX by SIR (selected ion recording) using m/z 316 as the mass determinant and 5% glycerol, 0.5% acetic acid, 0.025% sodium dodecyl sulfate, 0.1% PEG-400 and 0.5% PEG-300 as the matrix. (A) Multiple injections of 50ng of Neo-SXT. (B) Multiple injections of 5ng of Neo-SXT.

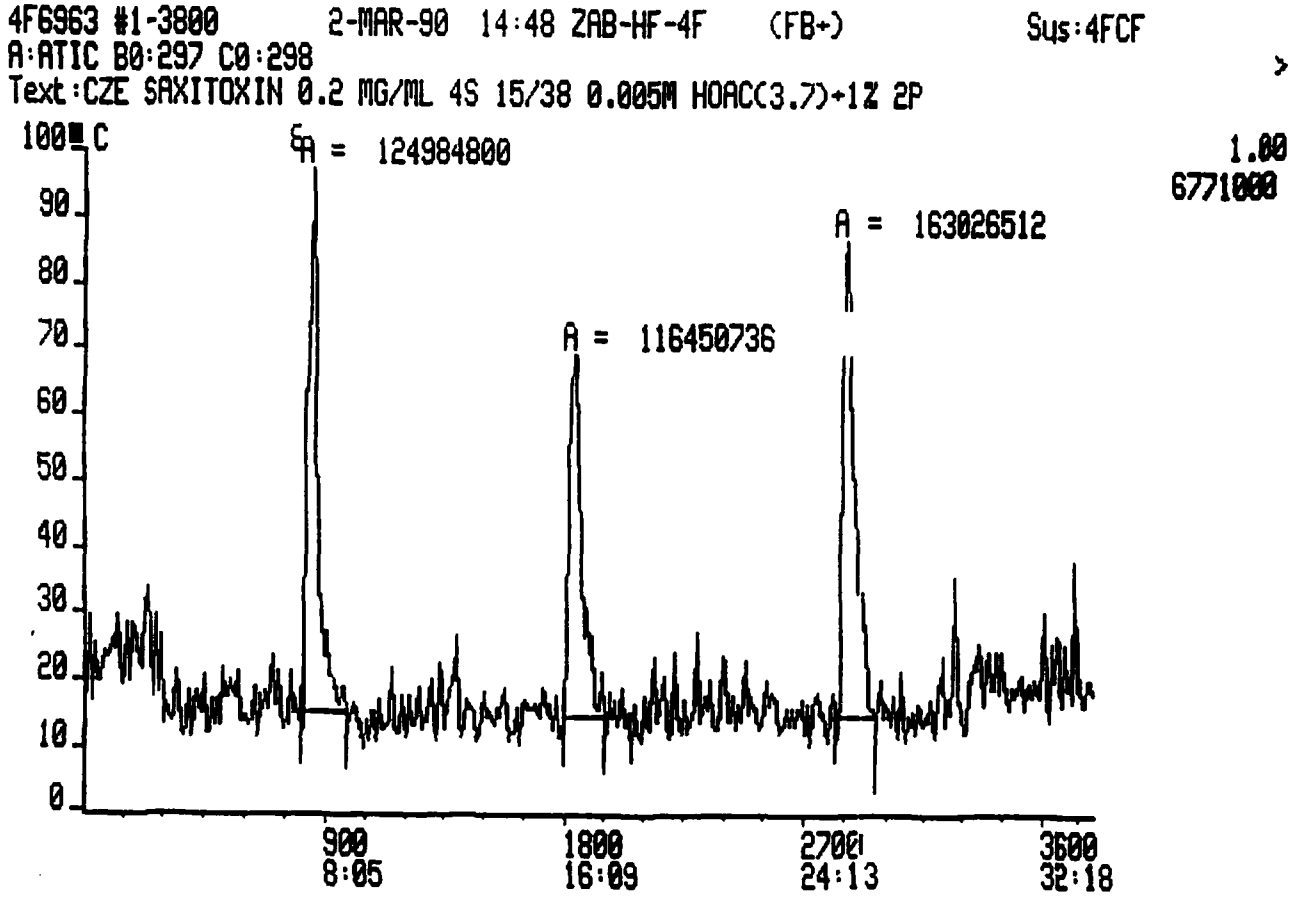


Figure 21 . Multiple injections of 80 pg of STX resolved by capillary Zone Electrophoresis (CZE) and detected by FAB. The matrix used was a 0.005M acetic acid buffer (pH 3.7).

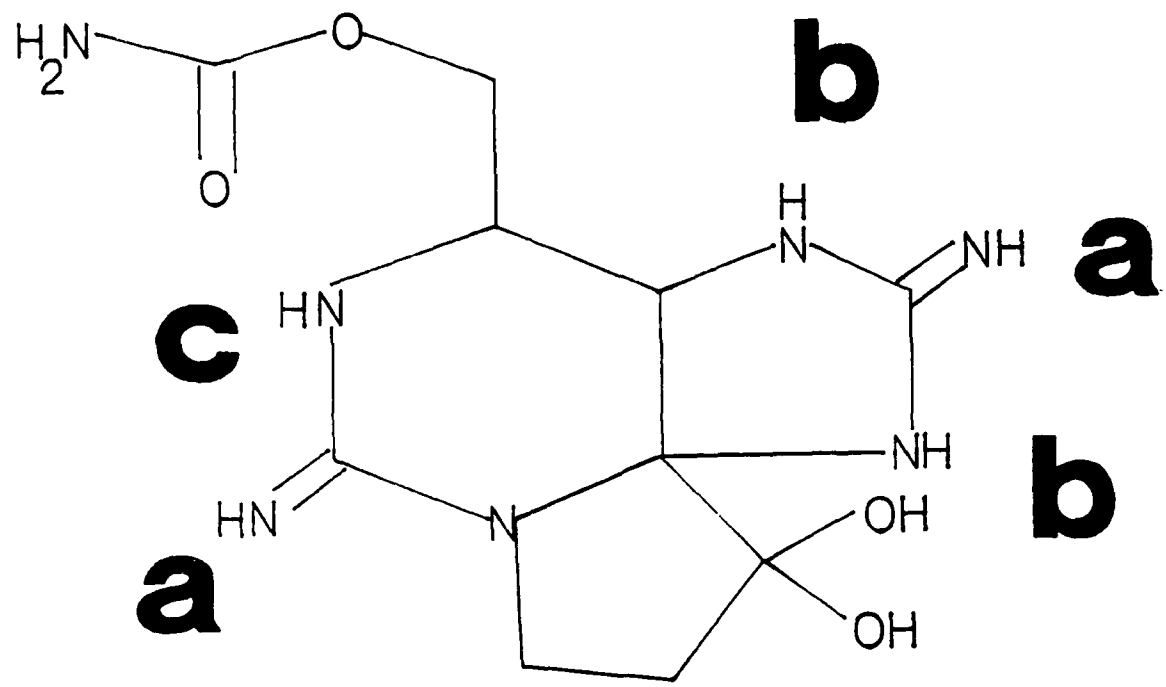


Figure 22. Positions of functional groups in saxitoxin.

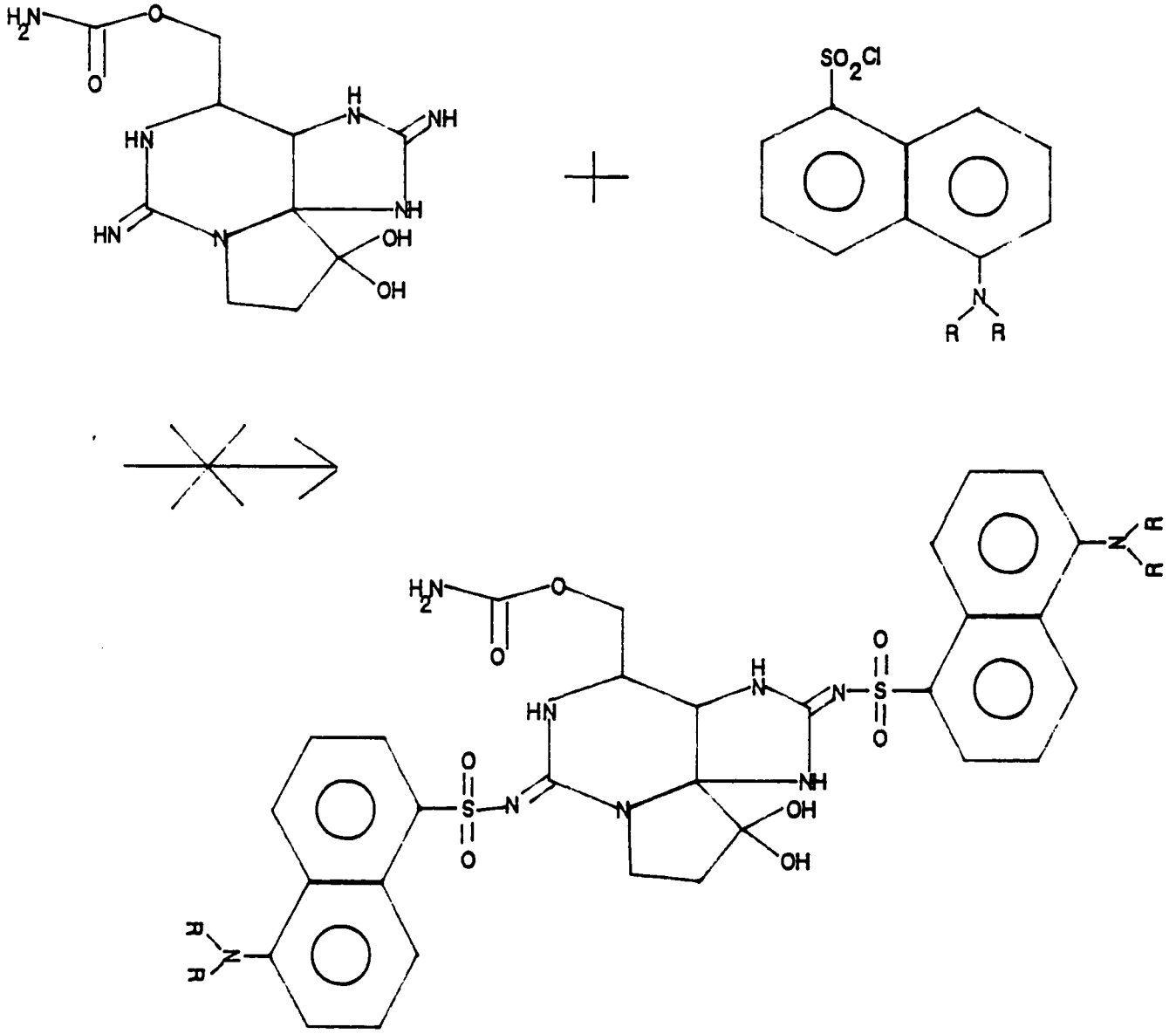


Figure 23. The expected reaction scheme for dansylation of saxitoxin.

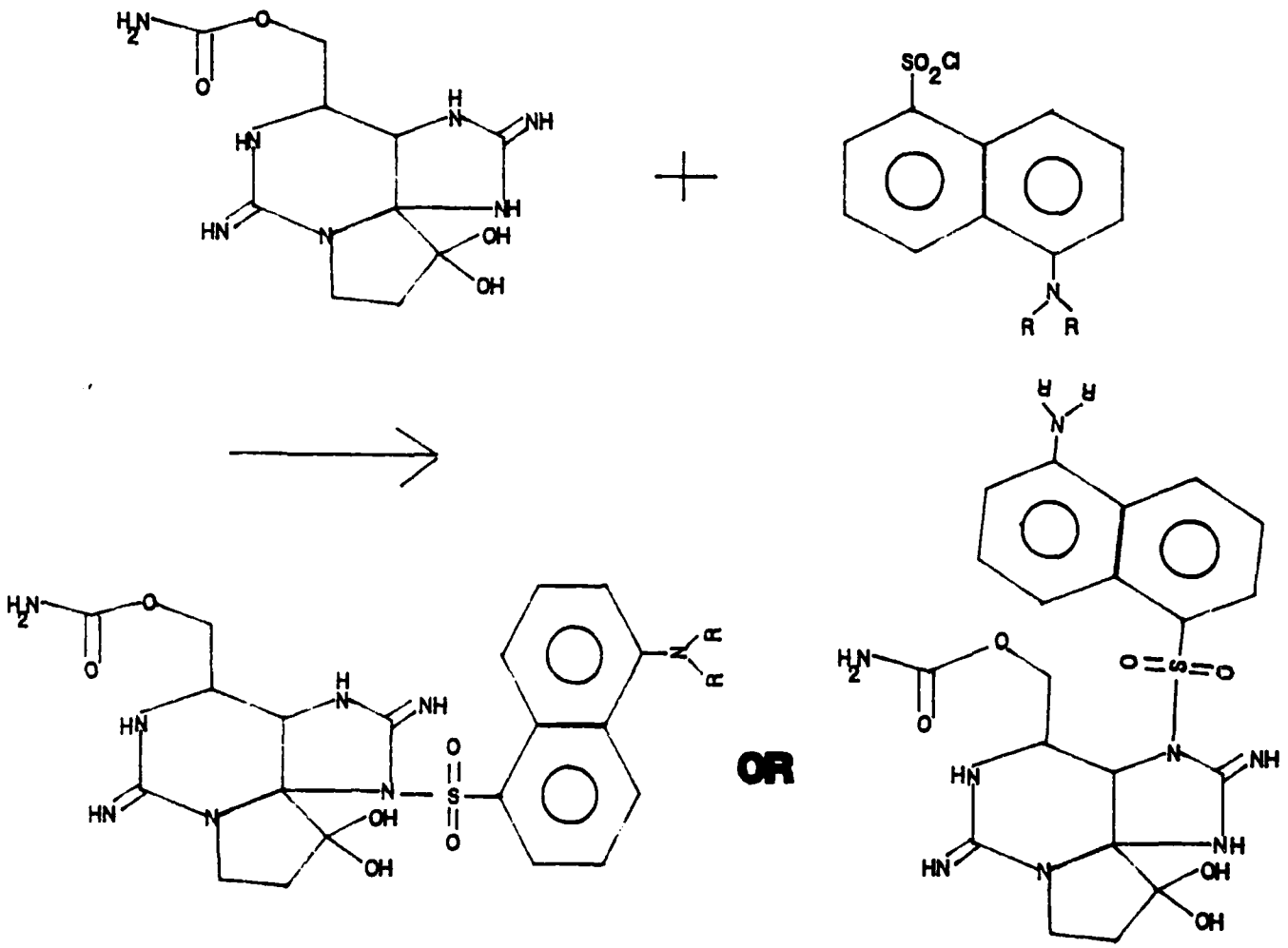


Figure 24. The suggested reaction scheme for dansylation of saxitoxin.

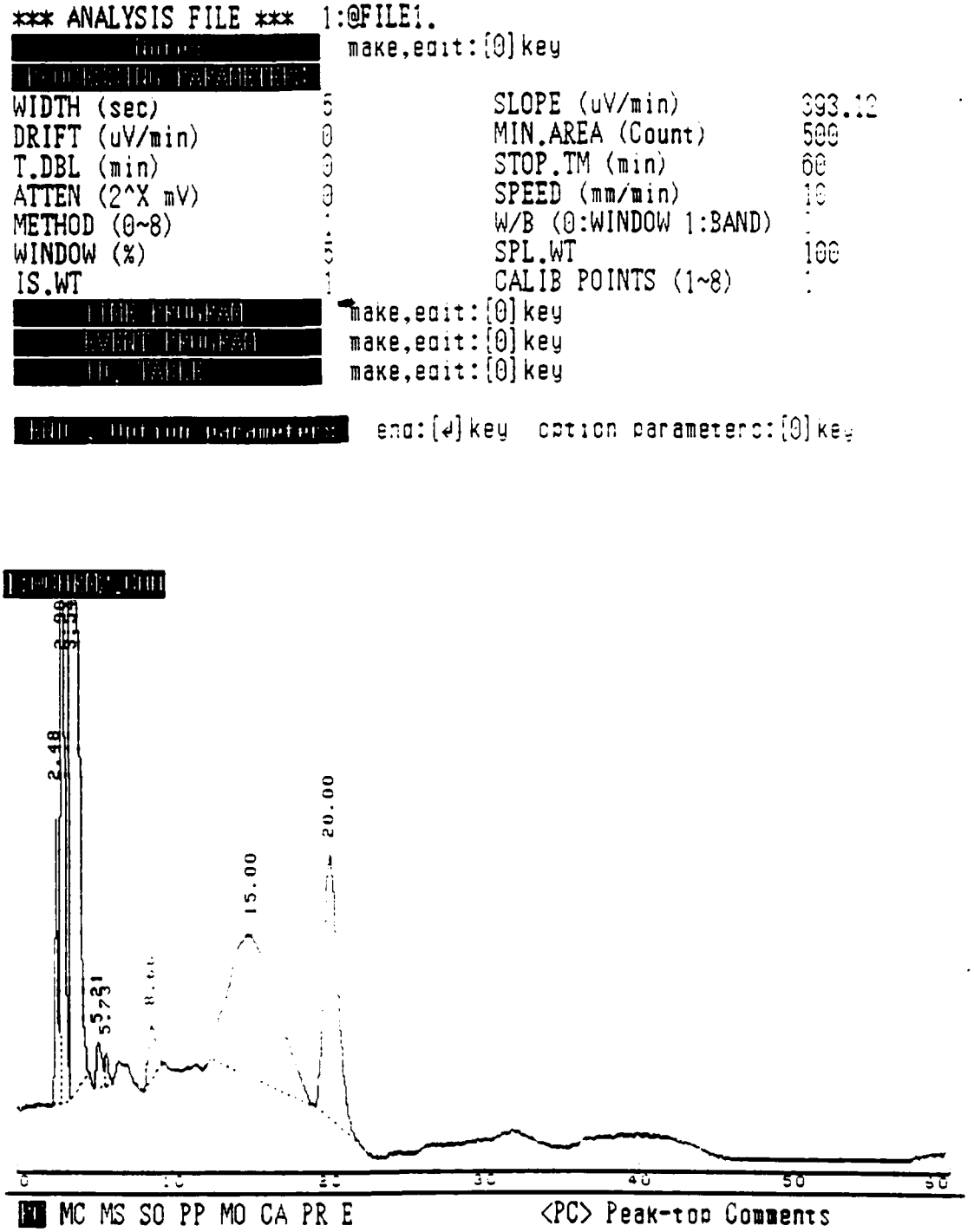


Figure 25. Resolution of the reaction products and reagents involved in the dansyl chloride reaction with STX. The conditions and column used are described in the top file whereas the actual separation is in the lower box. The peak at retention time 8.66 is the reaction product which is visible on TLC as well. The major components are dansyl chloride reagents used.

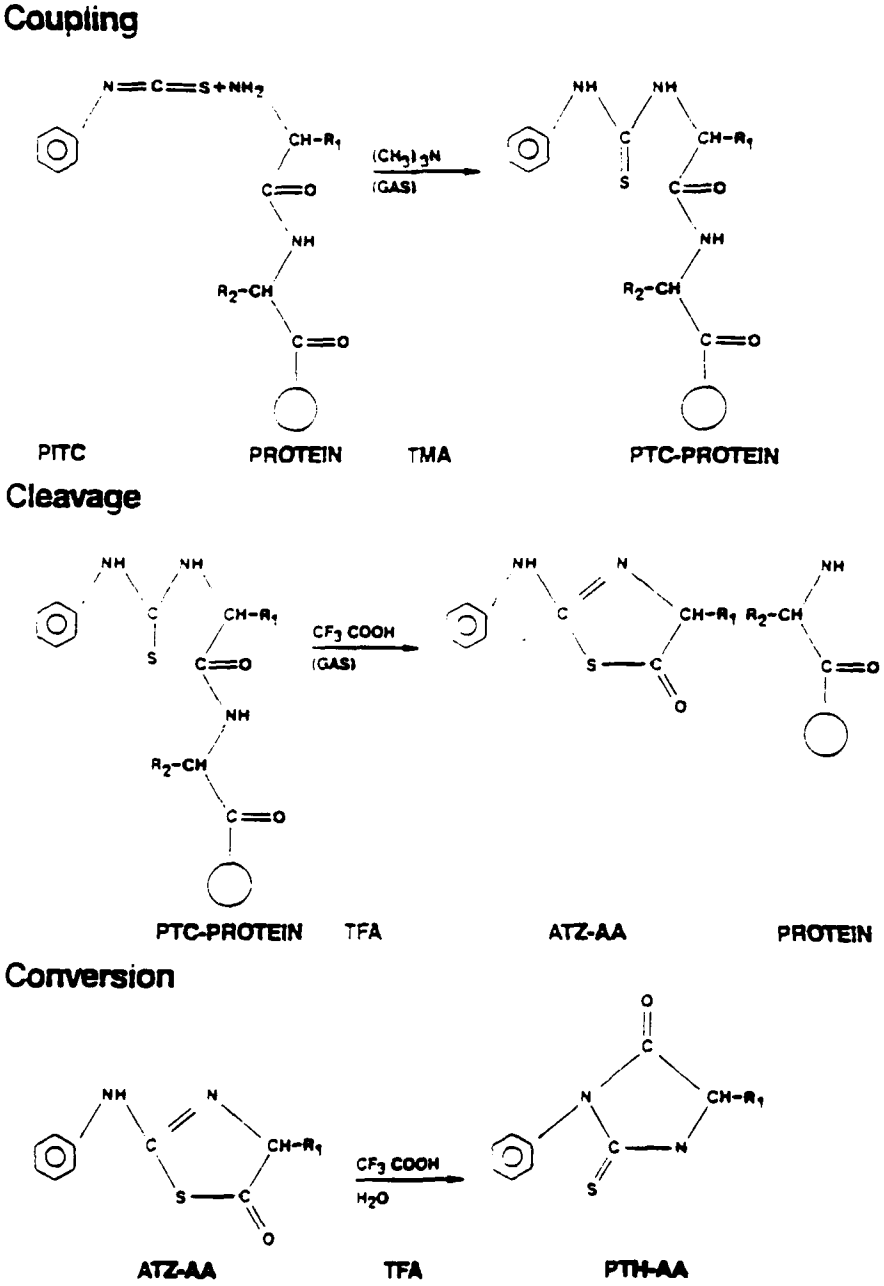


Figure 26 . Edman reaction involving nitrophenylisothiocyanate with free amines. This reaction is actually used for sequencing of amino acids from proteins. However, we speculated that perhaps the reaction with the free carbamoyl nitrogen of STX would be useful in analysis. The diagram is taken from Shively, J.E. 1986. Methods of Protein Microcharacterization, Humana Press, Clifton, NJ.

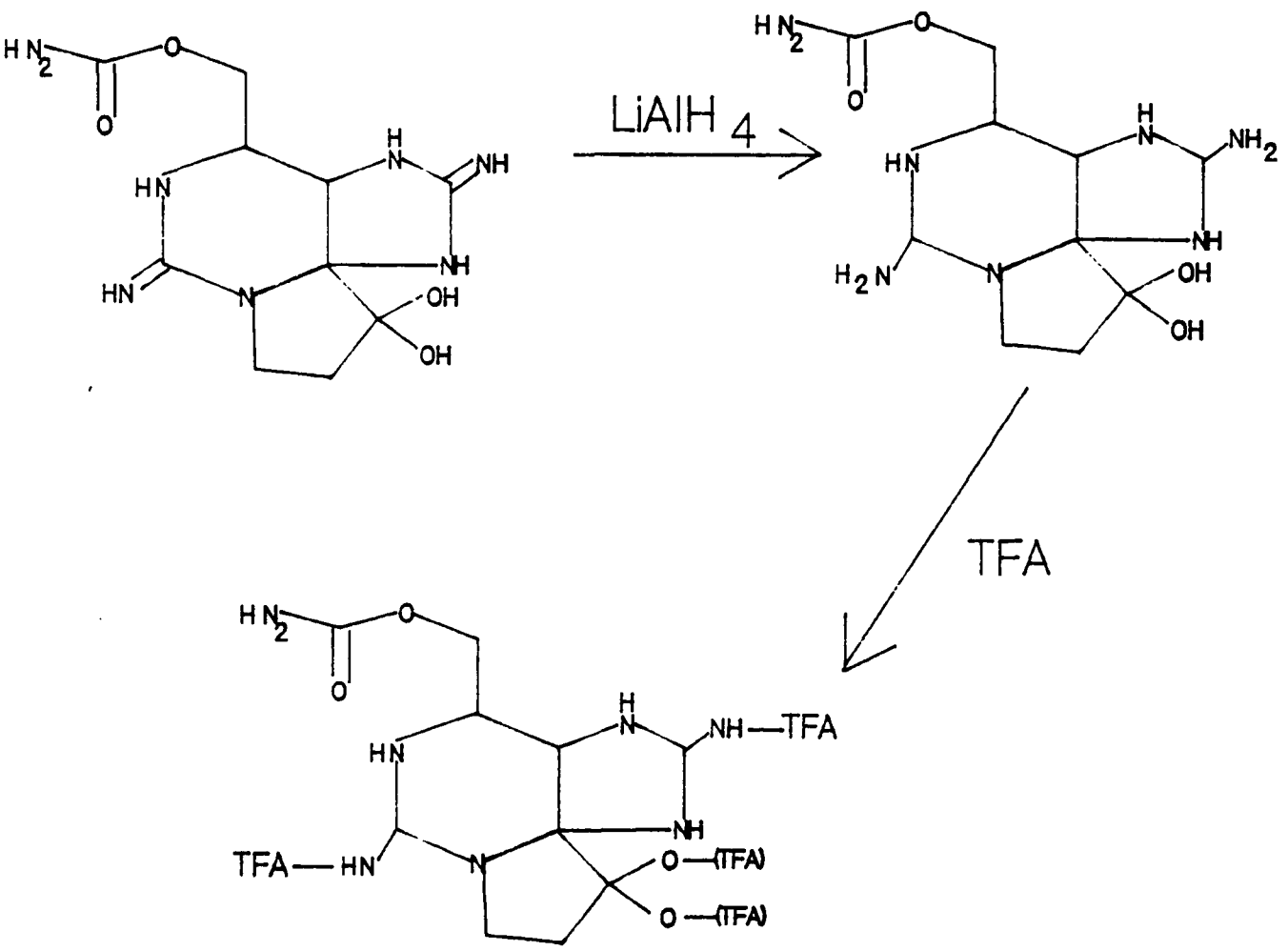


Figure 27. The hypothetical derivatization scheme of saxitoxin for the reaction of LIALH4 and TFA.

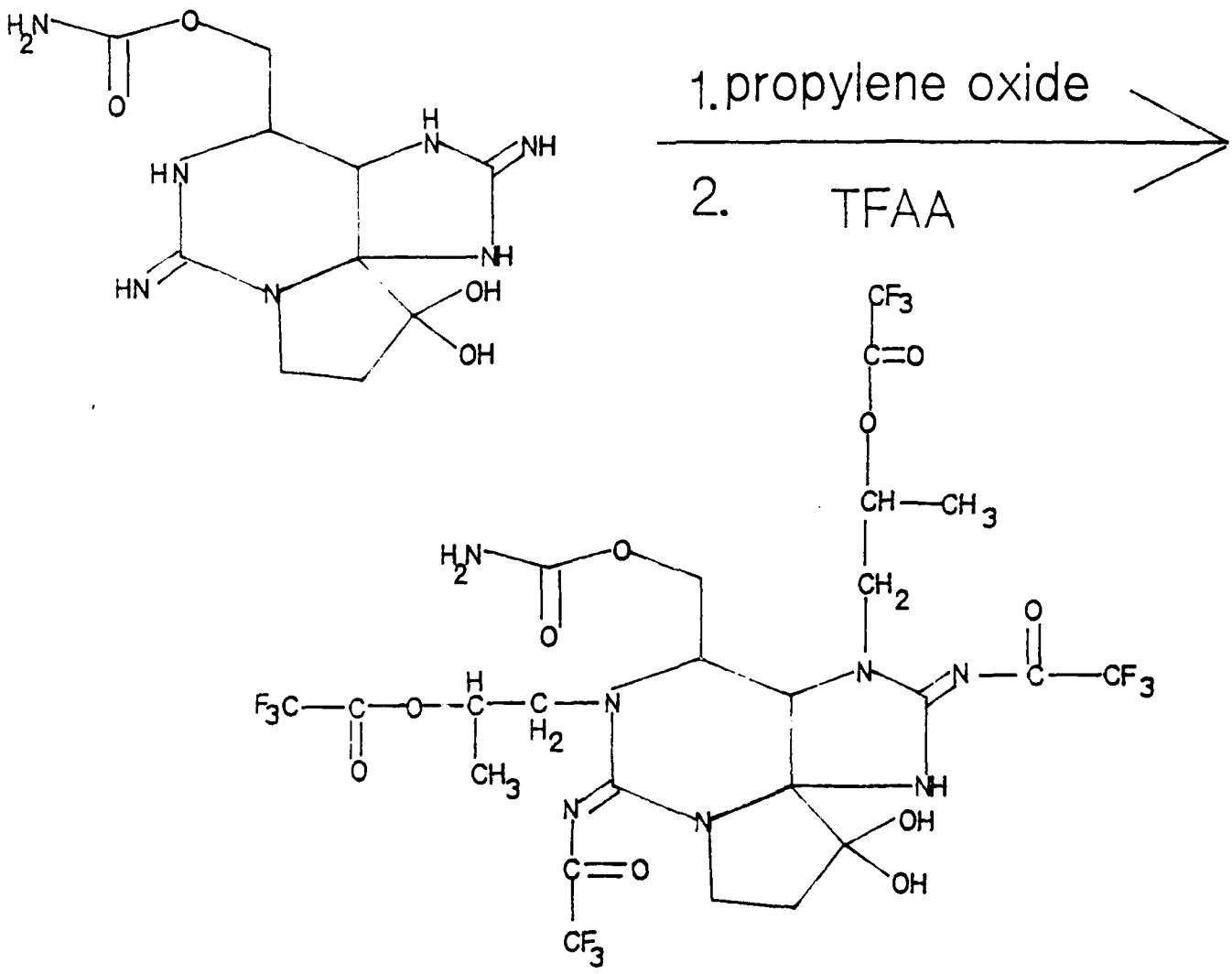


Figure 28. The expected reaction scheme of saxitoxin for the reaction of saxitoxin with propylene oxide and TFAA.

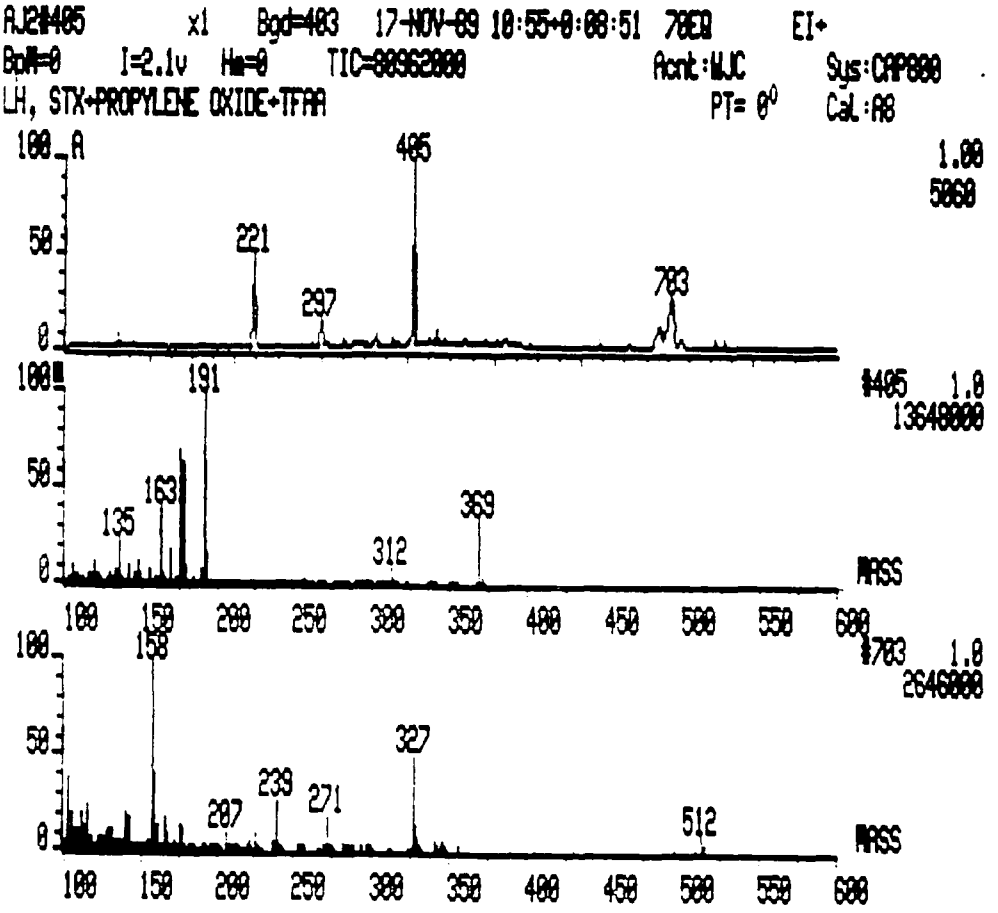


Figure 29 . Mass spectra of the reaction products of STX and propylene oxide and trifluoroacetic anhydride. The separation was done by gas chromatography (250 micron column) and the effluent monitored by electron impact mass spectrometry. Four chromatographic peaks were detected all of which were present in the control matrix i.e. the reagents minus the STX. Scans 405 and 703 are shown for inspection. None of the spectra match the expected values.

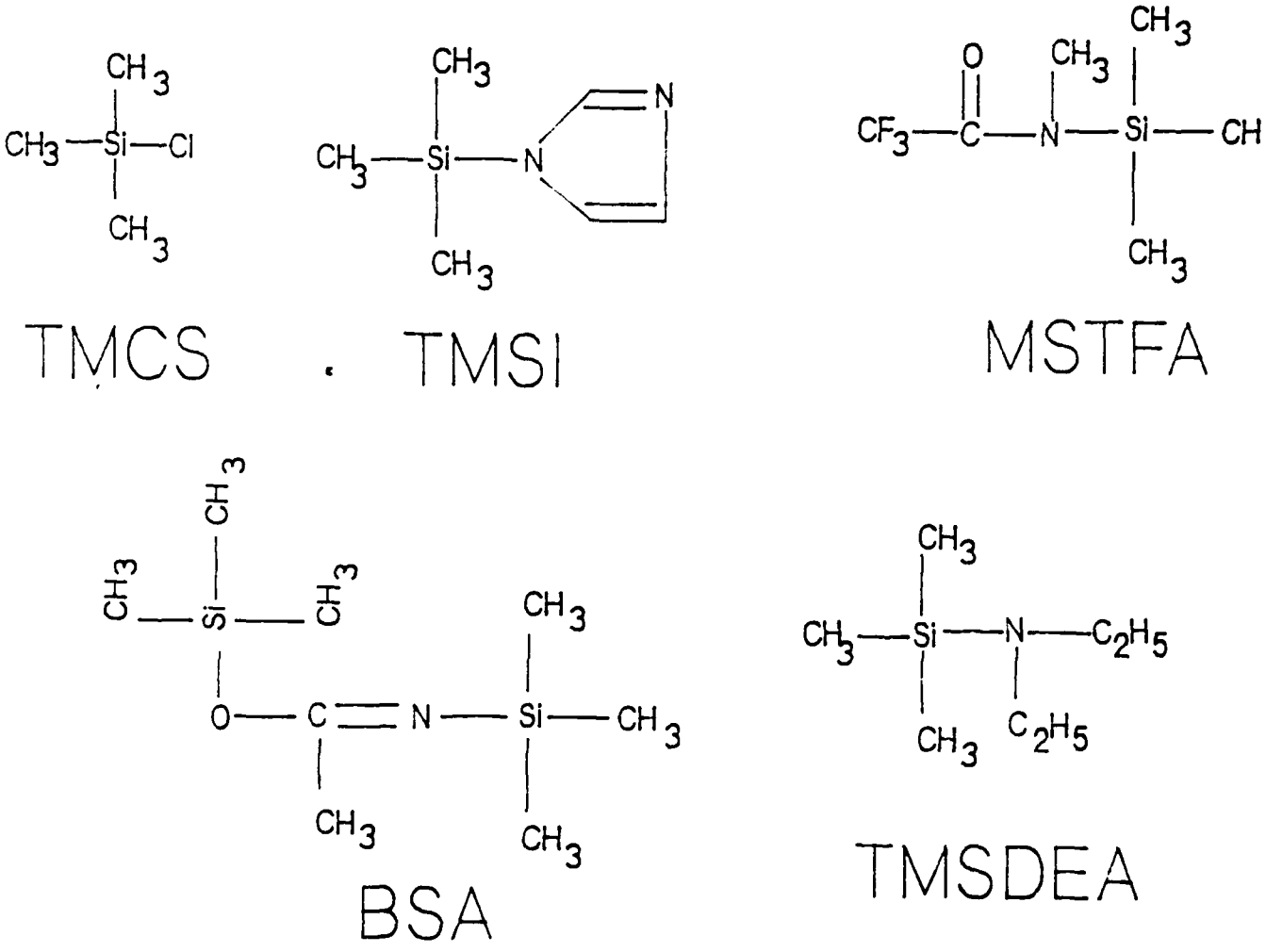
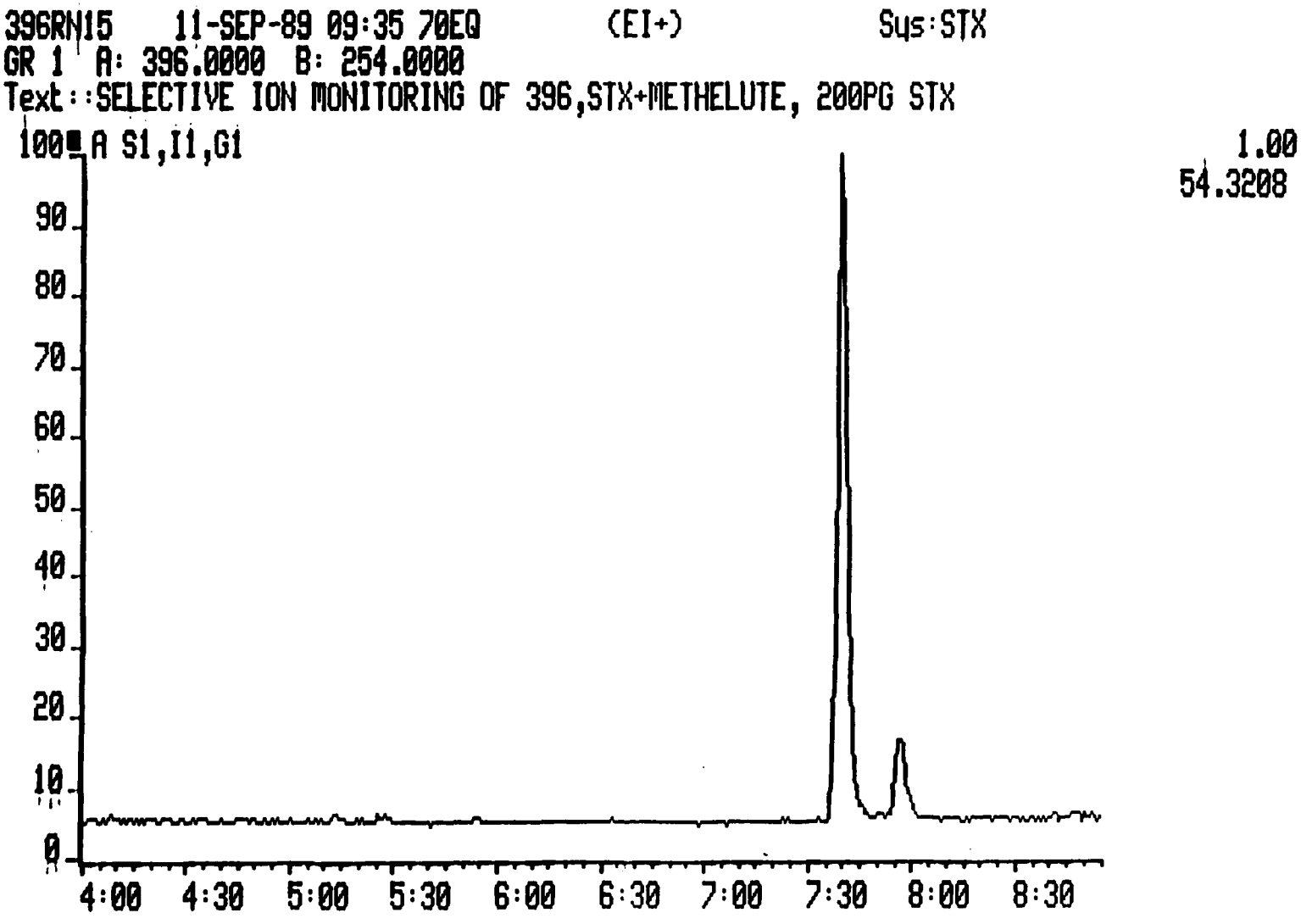


Figure 30. The molecular structures of the silylating reagents.

Figure 31 . Selective ion recording of m/z 396 of the product obtained from reaction of SXT with Methelute. The full scale deflection represents ~200 picograms of the reaction product.



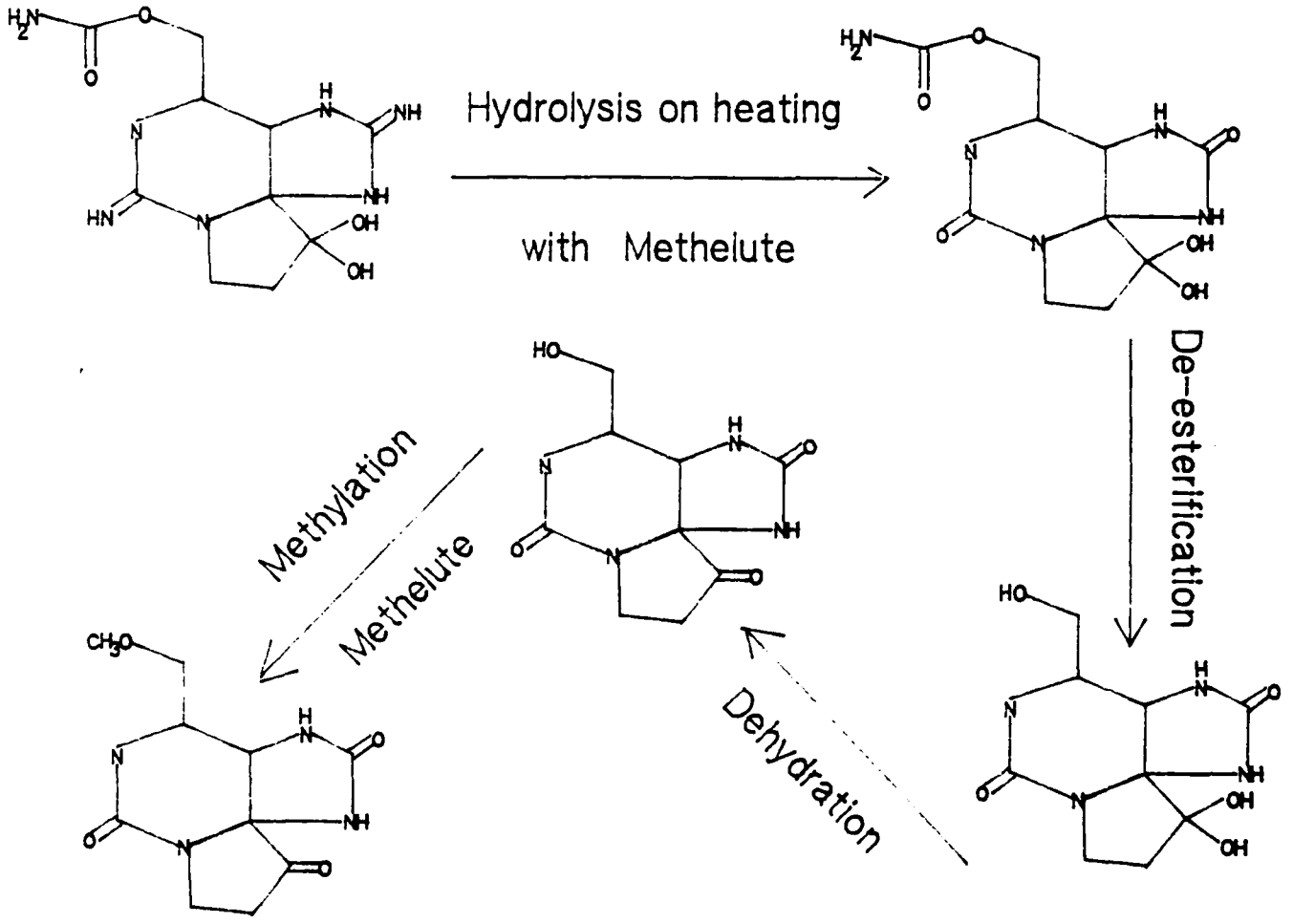


Figure 32. The hypothetical reaction scheme for the formation of the component of M=254.

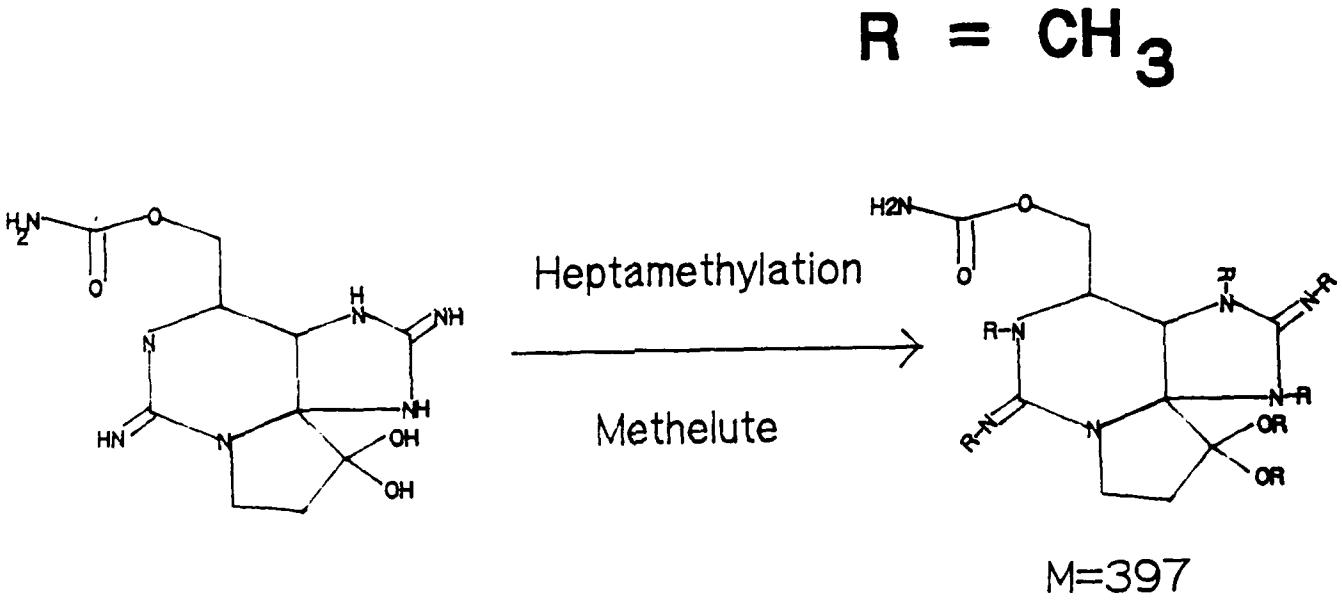


Figure 33. The hypothetical reaction scheme for the formation of the component of M=396(397-1).

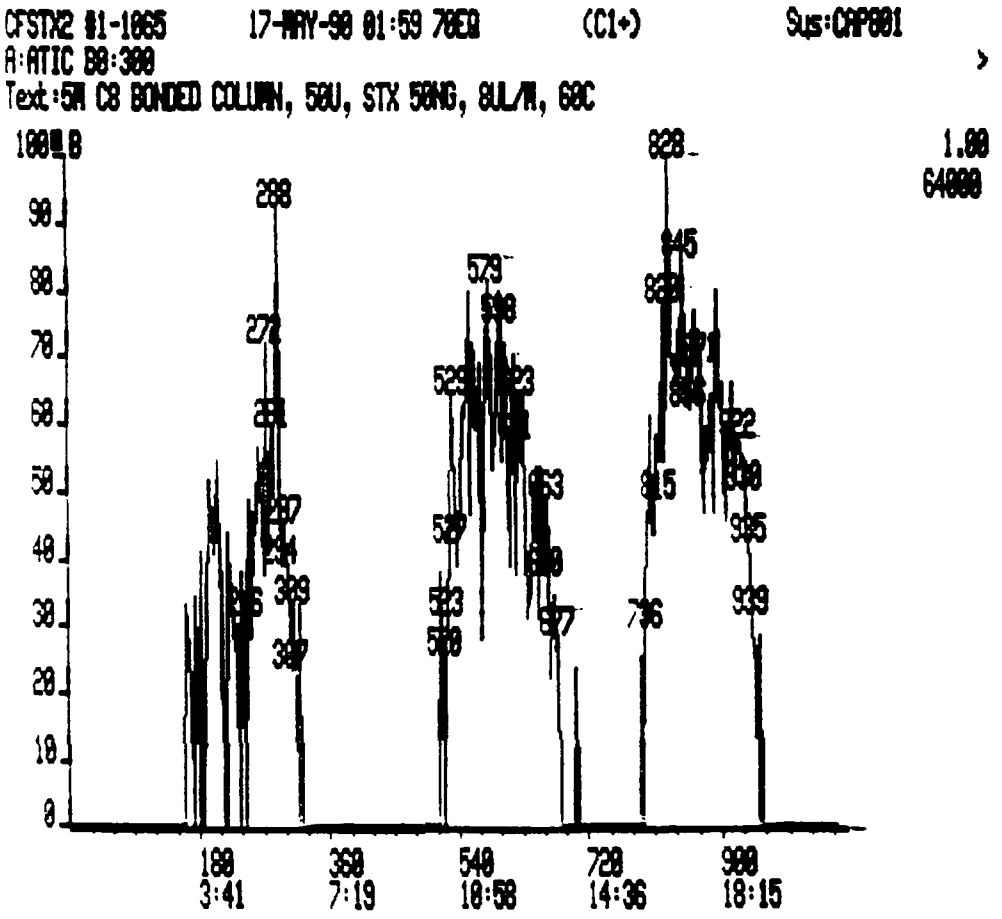


Figure 34 . Multiple injections of 50ng STX through a bonded capillary column (C-8, 5m, 50 micron I.D.).

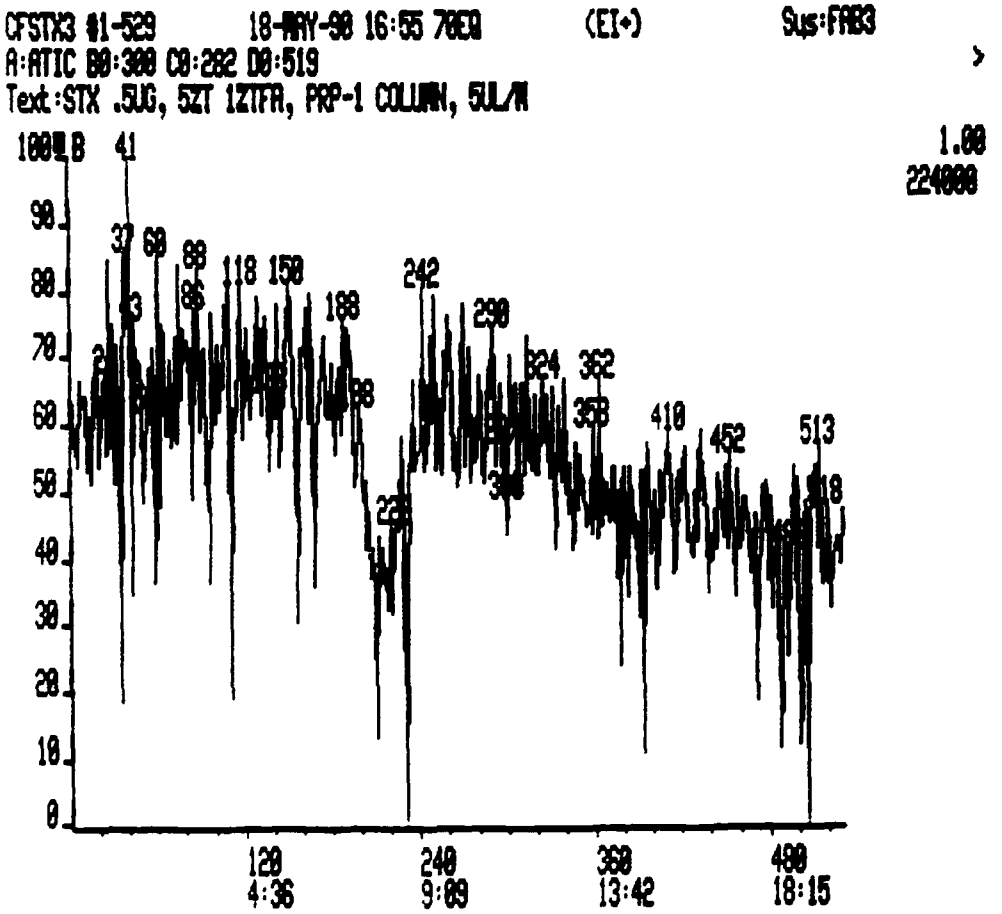


Figure 35. Multiple injections of 500ng STX through a packed capillary column (20cm, 530µ I.D., stationary phase: 10µ Hamilton PRP-1).

Literature Cited

1. Schantz, E.J., Mold,J.D., Stranger,D.W., Shavel,J., Riel,F.J., Bowden,J.P., Lynch,J.M., Wyler,R.S., Riegel,B. and Sommer,H. (1957), J. Amer. Chem. Soc. 79:5230-5235.

2. Hashimoto,K. and Noguchi,T. (1989) Recent Studies on Paralytic Shellfish Poison in Japan. Pure & Appl. Chem. 61:7-18.

3. Sommer,H., Whedon,W.F., Kofoid,C.A. and Stohler,R. (1937) Arch. Pathol. 24:537-559.

4. Schantz,E.J., Ghazarossian,V.E., Schnoes,H.K., Strong,F.M., Springer,J.P., Pezzanite,J.D., and Clardy,J. (1975) The structure of saxitoxin. J. Amer.Chem. Soc. 97:1238-1239.

5. Schantz, E.J. (1986) Chemistry and biology of saxitoxin and related toxins. Ann. N. Y. Acad. Sci. 479:15-23.

6. Schantz, E.J. (1970) The Dinoflagellate poisons. Chapter1 in :Microbial Toxins, Vol VII, Algal and Fungal Toxins. Eds. Kadis,S., Ciegler,A., Aji,S.J. Academic Press, New York.

7. Martin, D.F. and PAdilla, G.M. (1973) Marine Pharmacognosy -- Action of Marine Biotoxins at the Cellular Level. Academic Press, New York.

8. NAkamura,M., Oshima,Y., and Yasumoto,T. (1984) Occurence of saxitoxin in Puffer fish. Toxicon 22:381-385.

9. Shimizu,Y. (1986) Chemistry and biochemistry of saxitoxin analogues and tetrodotoxin. Ann. N.Y. Acad. Sci. 479:24-31.

10. Shimizu,Y., Kobayashi,M., Genenah,A., and Ichihara,N. (1984) Boisynthesis of paralytic shellfidh toxins in: Seafood Toxins ACS Symposium Series Ed. Ragelis,E.P. Amreican Chemical Society, Washington D.C. pp 151-160.

11. Koehn,F.E., Ghazarossian,V.E., Schantz,E.J., Schnoes,H.K., and Strong,F.M. (1981) Derivatives of saxitoxin. Bioorg. Chem. 10:412-428.

12. Shimizu,Y., Hsu,C., and Genenah,A. (1981) Structure of saxitoxin in solutions and stereochemistry of dihydrosaxitoxins. J. Amer. Chem. Soc. 103:605-609.

13. Bordner,J., Thiessen,W.E., Bates,H.A., and Rapport,H. (1975) The structure of a crystalline derivative of saxitoxin. The structure of saxitoxin. J. Amer,Chem. Soc. 97:6008-6012.

14. Noccolai,N., Schnoes,H.K., and Gibbons,W.A. (1980) Study of the stereochemistry, relaxation mechanisms, and internal motions of

natural products utilizing protn relaxation parameters: Solution and crystal structures of saxitoxin. J. Amer. Chem. Soc. 102:1513-1517.

15. Tanino,H., Nakata,T., Kameko,T., and Kish,Y. (1977) A stereospecific total synthesis of d,l-saxitoxin. J.Amer. Chem. Soc. 99:2818-2819.

16. Maruyama,J., Noguchi,T., MAtsunaga,S., and Hashimoto,K. (1984) Fast atom bombardment and secondary ion Mass Spectrometry of paralytic shellfish poisons and tetrodotoxin. Agric. Biol. Chem. 48:2783-2788.

17. White,K.D., Sphon,J. and Hall,S. (1986) Fast atom bombardment Mass Spectrometry of 12 marine toxins isolated from protogonyaulax. Anal. Chem. 58:562-565.

18. Sullivan,J.J. and Wekell,M.M. (1984) Determination of paralytic shellfish poisoning toxins by high pressure liquid chromatography in seafood toxins. ACS Symposium Series, Ed. Edward P. Ragelis, American Chemical Society, Washington D.C. pp 197-205.

19. Sullivan,J.J., Wekell,M.M., and Kentala,L.L. (1985) Application of HPLC for the determination of PSP toxins in shellfish. J. FOOD. Sci. 26:1506-1516.

20. Oshima,Y. Machida,M, Sasaki,K., Tamaoki,Y., and Yasumoto,T. (1984) Liquid chromatographic-fluorometric analysis of paralytic shellfish toxins. Agric. Biol. Chem. 48:1707-1711.

21. Rubinson,K.A. (1982) HPLC separation and comparative toxicity of saxitoxin and its reaction products. Biochim. Biophys. Acta 687:315-320.

22. Onoue,Y., Noguchi,T., Nagashima,Y., Hashimoto,K., Kanoh,S., and Tsukada,K. (1983) Separation of tetrodotoxin and paralytic shellfish poisons by high performance liquid chromatography with a fluorometric detectin using o-phthaldehyde. J. chromatogr. 257:373-379.

23. Mosley,S., Ikawa,M., and Sasner,J.J. (1985) A combination fluorescence assay and Folin-Ciocalteau phenol reagent assay for detection of paralytic shellfidh poisons. Toxicon 23:375-381.

24. Yentsch,C.M (1981) Flow cytometric analysis of cellular saxitoxin in the Dinoflagellate Gonyaulax Tamarensis var. Excavata. Toxicon 19:611-621.

25. Davio,S.R., Foltelo,P.A. (1984) A competitive displacement assay to detect saxitoxin and tetrodotoxin. Anal. Biochem. 11:199-204.

26. Quilliam,M.A., Thomson,B.A., Scott,G.J., and MichaelSiu,K.W. (1989) Ion-spray Mass Spectrometry of marine neurotoxins. Rapid. Comm. Mass. Spect. 3:145-150.

27. Wright,B.W., Ross,G.A., Smith,R.D. (1989) Capillary zone / laser fluorescence electrophoresis with laser fluorescence detection of marine toxins. J. Microcolumn Sep. 1:85-89.

28. Caprioli,R.M. (1988) Continuous flow FABMS -- An overview. 36th ASMS conference on Mass Spectrometry Proceedings. pp 729-730.

29. Cerny,R.L., Hayes,R.N., and Gross,M.L. (1988) Tandem mass spectrometry of biomolecules by using cintinuous flow FAB. 36th ASMS conference on Mass Spectrometry Proceedings pp 737-738.

30. Wekell,M.M. (1990) Seafood Toxins (General Referee Reports). J. Assoc. Off. Anal. Chem. 73:113-117.

31. Capriol,R.M., Dague,B., Fan,T., and Moore,W.T. (1987) Microbore HPLC / Mass Spectrometry for the analysis of peptide mixtures using a continuous flow interface. Biochem. Biophys. Res. Comm. 146:291-299.

32. Caprioli,R.M. (1990) Continuous flow fast atom bombardment Mass Spectrometry. Anal. Chem. 62:477A-485A.

33. Caprioli,R.M., Moore,W.T., Dague,B, and Martin,M. (1988) Microbore high performance liquid chromatography - Mass Spectrometry for the analysis of proteolytic digests by continuous flow fast atom bombardment Mass Spectrometry. J. Chromatogr. 443:353-362.

34. De Wit,J.S.M., Deterding,L.J., Moseley,M.A., Tomer,K.B., and Jorgenson,J.W. (1988) Design of a coaxial continuous flow fast atom bombardment probe. Rapid Comm. Mass. Spect. 2:100-104.

35. Moseley,M.A., Deterding,L.J., De Wit,J.S.M., Tomer,K.B, Kennedy,R.T., Bragg,N, and Jorgenson,J.W. (1989) Optimization of a coaxial continuous flow fast atom bombardment interface between capillary liquid chromatography and magnetic sector Mass Spectrometry for the analysis of biomolecules. Anal. Chem. 61:1577-1584.

36. Deterding,L.J., Moseley,M.A., Tomer,K.B., and Jorgenson,J.W. (1989) Coaxial continuous flow fast atom bombardment in conjuction with tamdem Mass Spectrometry for the analysis of biomolecules. Anal. Chem. 61:2504-2511.

37. Mosher,H.S. (1986) The chemistry of tetrodotoxin. Ann. N.Y. Acad. Sci. 479:32-43.

38. Sheers,E.H., (1966) Guanidine and guanidine salts. Kirk-Othmer

Encycl. Chem. Technol. 2nd Ed. 10:734-740.

39. Dunges,W., Naudorf,G., and Seiler,N. (1974) High pressure liquid chromatographic analysis of barbiturates in the picomole range by fluorometry of their DANS'derivatives. J. Chromatogr. Sci. 12:655-657.

40. Schmidt., G.J., Adams,R.F., Vandermark,R.F., and Slavin,W. (1977) Pre-column dansyl chloride derivatization for liquid chromatography with fluorescence detection. Chromatographic News Letter. 5:33-36.

41. Schmidt,G.J., Olson,D.C., and Slavin,W. (1979) Amino acid profiling of protein hydrolysis using liquid chromatography and fluorescence detection. J. Liq. Chromatogr. 2:1031-1045.

42. Ross,M.S.F, (1977) Determination of metformin in biological fluids by derivatization followed by high performance liquid chromatography. J. chromatogr. 133:408-411.

43. Bjorkhem,i., Blomstrand,R., and Ohman,G. (1977) Mass fragmentography of creatinine proposed as a reference method. Clin. Chem. 23:2114-2121.

44. Ciucanu,I. and Kerek,F. (1984) A simple and rapid method for the permethylation of carbohydrates. Carbohydrate Res. 131:209-217.

45. Waeghe,T.J., Darvill,A.G., Mcneil,M., and Albersheim,P. (1983) Determination by methylation analysis of the glycosyl-linkage compositions of microgram quantities of complex carbohydrates. Carbohydrate Res. 123:281-304.

46. Shelly,D.C., Gluckman,J.C., and Novotny,M.V. (1984) Dead volume free termination packed column in microcapillary liquid chromatography. Anal. Chem. 56:2990-2992.

47. Novotny,M.V. (1988) Recent advances in microcolumn liquid chromatography. Anal. Chem. 60:500A-510A.