CHARACTERIZATION OF THE FATTY ACIDS IN THE LIPIDS OF SNAKE VENOMS

By

MARY ELLEN COOKE

Bachelor of Science

Southeastern Oklahoma State University

Durant, Oklahoma

1983

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1986

CHARACTERIZATION OF THE FATTY ACIDS IN THE LIPIDS OF SNAKE VENOMS

Thesis Approved:

George V. Odell
Thesis Adviser

Charlotte L. Darby

Andrew Mont

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

I would like to express my appreciation to the members of my advisory committee. I would like to especially thank Dr. Andrew Mort for the use of his gas chromatograph and for his assistance and also Dr. Charlotte Ownby for her advice and help in venom acquisition. I wish to express my sincere gratitude to Dr. George Odell without whom this work would not have been accomplished. As an adviser and friend he has been invaluable.

I would like to thank Dr. George Waller and Steve McGown for performing and interpreting mass spectra. I wish to acknowledge the assistance and friendship of Sue Ann Hudiburg, Jeffery Schatz, and Paul and Carol Swartz.

Grateful acknowledgement is extended to the Biochemistry Department for facilities and financial support.

A special thanks is extended to my father, who is always with me, and to my mother for support both financial and emotional. I would also like to say "thanks" to the rest of my family for being there.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

In the United States around forty five thousand people are bitten annually by snakes(1). Of these bites eight thousand are from venomous snakes, with seven thousand people exhibiting some symptoms of envenomation. Twelve to twenty people die either due to the effect of the venom itself or treatment adminstered because of the bite. This is a much more serious problem in other parts of the world. In Asia more than twenty-five hundred people die per year from snake bites and in Costa Rica 2.2 per cent of the population is bitten by snakes with 0.5 per cent of the 2.2 per cent dying from the bite (2).

The symptoms of envenomation vary. Some venoms produce mainly systemic effects such as hypertension, excessive bleeding, etc., with very little local effects such as swelling and necrosis, which are the major concerns of viper venoms. The reason for the variation in symptoms can be attributed to the difference in the composition of the venoms of the differing species of snakes. The major toxic component may be a hemorrhagic toxin, a neurotoxin, or a myotoxin.

Effective antivenoms have been prepared for several venoms, however, the cause of all toxic effects are far from being understood. Therefore, it is desirable to obtain a greater knowledge of the components that make up the venom of snakes, and their biological roles.

Proteins are the major component of venoms and the most widely studied, since they are thought to be responsible for biological activity. Other constituents of the venom include: smaller peptides, free amino acids, carbohydrates, amines, nucleotides and related compounds, metal ions, and lipids (3).

This research has been concerned with the lipid fraction of venoms. Lipids make up only a very small portion of the venom. There is no evidence which attributes the lipid fraction to any of the toxic activity present in the venom, however, it has been documented that the removal of the lipid fraction significantly decreases the toxicity of the venom (4,5). The reason for this is not yet known. Several possible explanations have been proposed. Among them is the theory that the removal of the lipid fraction makes the venom less lipid soluble and the venom does not pass through the lipid bilayer of the cell membranes as efficiently (4). Another possibility is that the lipids, particularily the charged phopholipids, act in a synergistic way with the toxic protein to increase it's activity (5).

Yet another consideration is that the fatty acids in the lipid may serve as precursors and directly as biologically active compounds. Phospholipases are found to be present in numerous snake venoms. The production of lysolecithins and salts of fatty acids with the action of phospholipases on membrane phospholipids furnish compounds for cell lysis and micelle formation. It is a also theoretically possible that the phospholipases are hydrolyzing arachidonic acid from the phospholipids in the venom which is then transformed into one of the biologically active eicosanoids.

The major objective of this research was to identify the fatty acids present in snake venom.

The venom used for most of this research was that of C. atrox, the western diamond back rattlesnake. To identify the fatty acids in C. atrox venom the techniques of both packed column and capillary column gas chromatography were utulized as well as mass spectrometry.

Another goal of this research was to separate the lipids of the venom into their charged and neutral moities and then examine the fatty acids in the different lipid classes. Packed and capillary column gas chromatography was again used for the identification of the fatty acids. Thin layer chromatography was also used to determine what lipids were present.

The fatty acids of the lipid classes in the venom of Crotalus viridis viridis were identified and compared to the fatty acids in the lipid classes found in the venom gland, liver, and adipose tissue of Crotalus viridis viridis. Identification of the fatty acids was determined by capillary gas chromatography.

A comparision of the fatty acids in venoms of the following species of snakes was established: Crotalus adamanteus, eastern diamond back rattlesnake, Crotalus atrox, western diamond back rattlesnake, Bothrops asper, Central American viper, Agkistrodon c. contortrix, southern copperhead, Sistrurus miliarius baribouri, pigmy rattlesnake, Agkistrodon p. piscivorus, cottonmouth, Crotalus h. horridus, timber rattlesnake, Agkistrodon c. mokasen, northern copperhead. For the identification of these fatty acids capillary column gas chromatography was used.

CHAPTER II

LITERATURE REVIEW

Limited research has been conducted on the venom lipids of snakes. Consequently there exists only a small amount of literature on this subject. This may be due in part to the presumption that the small amount of lipid present in venom renders it's role in the venom insignificant.

One of the earliest reports of lipids in snake venom comes from Ganguly and Malkana who in 1939 identified cholesterol and lecithin in the venom of the cobra (7). They based their findings on the staining of the lipids with Fett-Schwarz, Geigy and "rouge cerol" after separation of the venom by electrophoresis (8,9).

Grasset et al in 1956 used the same method to analyze the venom of V. aspis, V. russelli, Bitis Arientans, N. naja, N. flava, Sepedon haemachates, Bungarus fasciatus, V. ammodytes and B. jararaca (10). Grasset et al were primarily interested in the protein components, and the only mention of lipids was that one or two fractions were observed with the lipid stain, the amount of lipid was so small they could not ascertain if the fractions represented free lipid or lipoproteins. They did not elaborate on how many fractions were seen in which venoms or if in all venoms lipids were observed.

In 1968 Devi reported in a review on the constituents of snake venom, that Naja naja venom contained 2.5 per cent lipids, russell's viper contained 1.7 per cent lipids, and Crotalus terrificus terrificus contained

1.0 per cent lipid (11). These percentages were based upon a phosphorus determination on chloroform-methanol extracts of the venoms. These results come from unpublished research by Devi et al obtained in 1963. No further analysis of the lipids were reportedly carried out.

It wasn't until 1969 that an extensive study of the lipids of snake venom was published. This was conducted by Kabara and Fischer on the venom of Naja naja the cobra. In their publication they state they first extracted the lipids from the venom using a chloroform-methanol mixture according to the Folch et al procedure (4). They then separated the different fractions of the lipid extract using column chromatography. The components separated were: cholesterol esters, triglycerides, cholesterol, diglycerides, monoglycerides, and phospholipids. The major components were phospholipids containing 83.0 per cent of the total lipid. Cholesterol was the second most abundant lipid posessing 10.0 per cent of the total lipid. The lipid components were then analyzed for their fatty acid content. They used both polar and non-polar packed column gas chromatography for the identification of the fatty acids. The major fatty acid was found to be arachidonate being present as 59.0 per cent of the fatty acids in the phospholipids. Arachidonic acid was also present in the neutral lipids as 7.0 per cent of the fatty acids, however, the neutral lipids contained more arachidic, stearic, and behenic acid, whose percentages are 24.0, 21.0 and 13.0 per cent respectively.

Kabara and Fischer tested the lipid for toxicity by injecting the lipid fraction into mice. The lipids by themselves were not found to be lethal. The removal of the lipids, which comprises 0.4 per cent of the venom, caused a loss of 40.0 to 50.0 per cent in lethality of the delipidated venom when injected into mice. They were unable to regenerate this lost toxicity by addition of the lipids back to the venom.

Similiar results were obtained by Morroz et al in 1966 who were characterizing the venom from Vipera palestinae, the palestenian viper (5). They also observed a decrease in toxicity with the removal of the lipid from the venom. Morroz et al demonstrated that lipids had an enhancing effect upon the toxicity of the venom. When lipid was added to the purified protein of the venom the toxicity increased four fold, lowering the lethal dose from 0.24 mg per mouse to 0.06 mg per mouse. Morroz et al were also able to regain the toxicity lost from the venom on removal of the lipids, by addition of the lipids back to the venom. An interesting finding by Morroz et al was the observation of only neutral lipids in the venom of Vipera palestinae with no indication of phospholipids.

Morroz et al made no attempt to explain the effect of the lipids on the toxicity of the venom. Kabara and Fischer, however, in the conclusion of their article made the following statement concerning the possible function of lipids in snake venoms:

> The role of lipids, therefore, in snake venoms is thus still open to speculation. One conjecture would be that a lipid toxin complex facilitates the body distribution and/or cellular penetration of the toxic peptide.

Researchers observing the activity of the venom when the lipids are and are not present provide further insight into the function of the lipids.

In 1941 McFarlane et al reported that when lipids were removed from plasma, the coagulating factor of Russell's viper venom did not coagulate the lipid free plasma (12). It was the speculation of these authors that the coagulating factor of Russell's viper venom must first form a lipid-coagulating factor complex before it could coagulate blood.

Again in 1949 Fault and Everard showed, in further coagulation studies of Russell's viper venom, that the thromboplastic activity of the venom

In 1955 Poole et al demonstrated that a "preparation of crude lecithin" accelerated the coagulation of blood by Russell's viper venom (14). The authors believed a free fatty acid to be responsible, however, neither sodium stearate or sodium oleate had an accelerating effect on the coagulation process. Poole and Robinson found in 1956 that the presence of phosphotidyl ethanolamine also enhanced the venom of Russell's viper in the clotting of blood (15).

More recent research by Bon et al on the venom of Crotalus durissus terrificus shows an increase in the phospholipase activity by the addition of negatively charged phospholipids (16, 6). The major toxic protein of C. durissus terrificus is crotoxin, a neurotoxin composed of two subunits, a basic, weak phospholipase A2 and a non-enzymatic acidic component. The non-enzymatic subunit while not being toxic itself, when bound to the phospholipase subunit increases the specificity and toxicity of the phospholipase. According to Bon et al this is a three step process in which in the third step negatively charged phospholipids bind to the subunit enhancing it's activity.

The findings of these latter authors indicate that though the lipid fraction is relatively small, it does play an active role in the toxicity of the venom. As more research is conducted on venom lipids and their function we may through the identification of what lipids are present in venom have better understanding of the mechanism of certain venoms.

CHAPTER III

MATERIALS AND METHODS

Materials

The standard fatty acids and fatty acid methyl esters were obtained from the following suppliers: Palmitic acid and stearic acid, Eastman Organic Chemicals, Rochester, New York; Arachidic acid, K & K Laboratories, Inc., Jamaica, New York; Behenic acid, Fluka, Switzerland; Methyl caprate, methyl laurate, methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate and methyl arachidate, Alltech Associated, Inc., Applied Science Laboratories, Deerfield, Illinois; Methyl linolenate and methyl arachidonate, Sigma, St. Louis, Missouri; Methyl linoleate, Applied Science Laboratories, Inc., Deerfield, Illinois.

The reagents used in the transesterification procedure include: Benzene, Burdick & Jackson Laboratories, Inc., Muskegon, Michigan; 2,2-Dimethoxy propane, Sigma, St. Louis, Missouri; Methanolic HCl kit, Alltech Associate, Inc., Applied Science Laboratories, Deerfield, Illinois.

The thin layer plates used in thin layer chromatography were 20 x 20 cm silica gel G plates purchased from Analtech Incorporated.

The C. atrox venom was collected in 1983 at the rattlesnake round-up in Sweetwater, Texas with the cooperation of Dr. John Perez from Texas A & I University, Kingsville Texas. The venom obtained from Perez was received as 400 ml of frozen liquid venom. Upon receipt of the venom, it was thawed centrifuged at a low speed and then freeze dried. The resulting

dried venom was approximately 100 grams. Bothrops asper venom comes from Costa Rica with the compliments of Dr. Luis Cerdas, Director of the Instituto Clodomiro Picado. Other venoms were purchased from the following companies: Crotalus adamanteus, Sigma, St. Louis, Missouri; Crotalus h. horridus, Ross Allen Reptiles Institute, Silver Springs, Florida; Agkistrodon c. contortrix and Agkistrodon p. piscivorus, Biotoxins Incorporated, St. Cloud, Florida; Sistrurus miliarius baribouri and Agkistrodon c. mokasen, Miami Serpentarium Laboratory. C. viridis viridis tissue was obtained at Oklahoma State University, Stillwater, Oklahoma, from four snakes in March of 1986. The C. v. viridis venom was also collected at Oklahoma State University, in August of 1986.

Methods

Procedures

Transesterification of Lipids. To increase the volatility of the fatty acids for gas chromatography the lipids were first derivatized to methyl esters. The transesterification method used was based upon the procedure described by Mason and Waller in 1964 (17). To each venom, standard fatty acid, or tissue sample the following reagents were added in the below order:

4.0 ml's sodium dried benzene

0.4 ml's 2,2-dimethoxy propane; mix

0.5 ml's methanolic HCl

Mix until only one phase is observed

The samples were then capped with aluminum foil and reacted for 12-24 hours. After completion of the reaction the upper lipid layer was pipetted off and dried under a stream of nitrogen gas. If extraction procedures

were performed prior to transesterification the sample was dried directly with nitrogen. Twenty to thirty ml's of hexane or benzene was added to the sample as solvent. The sample was then ready to be injected into the gas chromatograph. One microliter was usually injected.

Extraction of Triglycerides, Phospholipids, and Sphingolipids. An extraction of the venom lipids and snake tissue was performed to separate the lipid fraction into triglycerides, phospholipids, and sphingolipids for identification of their fatty acids. When extracting the snake tissues, the tissue was first homogenized with an Eherbech homogenizer. The extraction procedure is described below:

1. Add 10.0 ml's of acetone to the sample and stir for 5.0 minutes.
2. Vacuum filter the acetone mixture. Retain filtrate.
3. Repeat steps 1 and 2 on residual sample. Combine filtrates, label triglycerides and dry under nitrogen.
4. Repeat steps 1, 2 and 3 using a 2:1 chloroform-methanol mixture instead of acetone. Label combined filtrates phospholipids.
5. To the remaining sample add 15.0 ml's boiling ethanol. Stir 10.0 minutes.
6. Vacuum filter EtOH mixture. Retain filtrate and label sphingolipids. Dry under nitrogen.
7. To each lipid sample add approximately 1.0 ml of benzene and 1.0 ml of distilled H_2O.
8. Centrifuge each sample for 3.0 minutes. Pipette off upper lipid layer and save. Repeat 3 times combining lipid layers.
9. Dry samples under nitrogen.
10. Transesterify.

Extraction of Total Lipid. The total lipid components were extracted from various venoms, by the following procedure below:

1. Stir venom in diethyl ether for 5 minutes.
2. Vacuum filter diethyl ether mixture. Retain filtrate.
3. Repeat step 1 and 2 on remaining venom. Combine filtrates and dry under nitrogen.
4. Add 1.0 ml of benzene and 1.0 ml of H_2O to lipid extracts.
5. Centrifuge 3 minutes, piptette off upper lipid layer and save. Repeat 3 times.
6. Dry sample under nitrogen.
7. Transesterify.

Thin Layer Chromatography. Extracted lipid components were analyzed using thin layer chromatography. Four microliters of the sample being examined along with four microliters each of, sphyngomeylin/lecithin standard, monopalmitin, dipalmitin, tripalmitin standard, cholesterol standard, methyl ester fatty acid standard, were spotted five cm from the bottom of a 20 x 20 silica G TLC plate. The plate was developed for approximately three hours. The developing solvent was n-propanol:NH_4OH (70:30,V:V). After drying the plate was placed in an iodine chamber for visualization sprayed with molybdenum for phosphate identification.

Apparatus

Packed Column Gas Chromatograph. The packed column gas chromatograph was a Perkin Elmer 990. The column packing materials were 100/120 mesh chromasorb W, as the solid support, coated with 20% by weight diethylene glycol succinate (DEGS). The packing was prepared by dissolving the DEGS with an appropriate amount of acetone to make fluid then adding the mixture to 80.0 grams of chromasorb W. The resulting slurry was then rotary

evaporated until the majority of the acetone is removed. The remaining solvent was removed by a heated fluidized bed apparatus utilizing nitrogen gas. This produced a uniform coat of DEGS on the support. The G. C. column dimensions were 7.5ft. x 0.25in. o.d. The carrier gas was nitrogen, detection was by flame ionization. The injector temperature and manifold temperature was 250°C, the oven temperature was maintained isothermally at 180°C.

Capillary Column Gas Chromatograph. The capillary gas chromatograph was a Tracor 560. The column was a DB-1 fused silica column purchased from J & W Scientific, Inc. Dimensons were 30m x 0.25mm i.d. The carrier gas was helium, detection was by flame ionization. The samples were injected by a capillary injection system purchased from J & W Scientific, Inc., for on column injection. The oven temperature was programed for the sample to be injected at 75°C then raised and held at 180°C for four minutes, the temperature was then increased 3° per minute until it reached 230°C.

Mass Spectrometry. The LKB-2091 capillary gas chromatograph/mass spectrometer was used to obtain mass spectral data of the fatty acid methyl esters. The LKB-2091 is a unit resolution, magnetic sector instrument that has been upgraded. The manufactured G. C. oven has been replaced by a Packard 483ACGC oven and also the original jet separator assembly was removed and a capillary column installed directly to the ion source. The LKB-2091 is also equipped with an Teknivent data system based on the IBM-AT. This includes a library search of the data bases Wiley/NBS/EPA which were utilized. Two microliters were injected at a 1:5 split on a J & W Scientific, Inc., DB-5 column, the dimensions are 60m x 0.25mm with 1.0 micrometer film. The sample was injected at 100°C and held for four minutes,

increased by 10° per minute until 280 °C was reached and then held for 15 minute and flushed with 1 microliter of hexane.

Methods of Calculation for Fatty Acids

Calculation of the Relative % of Fatty Acids. the purpose of this research was a qualitative characterization of the lipids in venom. In characterizing the venom it was desirable to determine the relative per cent of fatty acids present. In order to calculate relative per cent the area of each fatty acid peak was established. This was accomplished when using the Perkin Elmer 990 by using the triangulation method (i.e. width at 1/2 height multiplied by peak height = area of triangle) on the peaks recorded by a printer based on detector response. The Tracor 560 gas chromatograph was interfaced to an Apple P.C. where a program based on detector response provided the area of each peak. The program takes several intervals along the response curve and multiplies the height of the interval by the elapsed time for the interval, the summation of all the products under the curve is the area of the curve. After the areas of the individual fatty acids were determined they are summed to give the total amount of fatty acids. The relative per cent of each fatty acid is then calculated by the following equation:

$$\frac{\text{Area of fatty acid}}{\text{Total areas of all fatty acids}} \times 100 = \% \text{ of fatty acid}$$

Statistical Analysis. In determining the retention time of the standard fatty acids several samples were injected into the G. C. and although the data was mostly consistent from run to run, the longer the fatty acid chain more variation was observed. The following standard deviation formula was performed on the standard fatty acids retention times and areas of unknown

fatty acids:

$$S = \sqrt{\frac{d_1^2}{N-1}}$$

S = standard deviation

d_1 = deviation from average $(x_i - \bar{x})$

N = number of sample

x_i = individual sample

$\bar{x}$ = average of samples

Due to the human error introduced in the manipulation of the gas chromatograph and recorder and also variations caused by changing columns the pattern of the eluting fatty acids as observed by the recorder as well as the recorded retention times of the fatty acids were considered in establishing the indentity of the fatty acids.

CHAPTER IV

RESULTS AND DISCUSSION

Identification of the Fatty Acids in Snake Venom

Retention Times of Standard Fatty Acid Methyl Esters

Prior to the identification of the fatty acids found in snake venom, retention times of standard fatty acids were established. Arachidic, stearic, and palmitic acids, in dilutions of 10.0 micrograms/ml, 5.0 micrograms/ml, 1.0 micrograms/ml, 0.5 micrograms/ml, 0.1 micrograms/ml, and 0.05 micrograms/ml, were transesterifiied and one microliter of each injected into the Perkin Elmer 990 packed column gas chromatograph. This was repeated five times. The average retention time of palmitic acid methyl ester was 5.85 minutes ± 0.29, stearic acid methyl ester was 10.66 minutes ± 0.10, and arachidic acid methyl ester was 19.70 minutes ± 0.15. These retention times were compared to the retention times of pre-methylated fatty acids purchased from Sigma. The purchased methyl palmitate, methyl stearate, and methyl arachidate were prepared in dilutions of 1.0 micrograms/ml, 0.5 micrograms/ml, 0.4 micrograms/ml, 0.2 micrograms/ml, 0.1 micrograms/ml, and 0.05 micrograms/ml. These fatty acid methyl esters were also injected five times each under conditions similiar to the previous transesterified fatty acids. The retention time of methyl palmitate was 5.8 minutes ± 0.23, methyl stearate was 10.8 minutes ± 0.10, and methyl arachidate was 21.1 minutes ± 0.20. There appeared to be little difference in the pre-methyl-

ated fatty acids and the fatty acids transesterified in the laboratory, therefore, the fatty acids purchased from Sigma were used in all further preparations of standards.

Fatty acid methyl ester standards ranging from octanoic to eicosatetraenoic acid methyl esters were injected into both capillary and packed column gas chromatographs. Table 1 shows the retention times of the standard fatty acid methyl esters injected singly and as a mixture into both the gas chromatographic instruments. These retention times are representative of a single injection. The standards had to be reinjected periodically on both columns due to the wear of the columns and after each repacking of the column. the chromatograms of the mixed fatty acid methyl esters are seen in Figures 1 and 2.

When using the Perkin Elmer 990 packed column gas chromatograph, retention times of fatty acid methyl esters increase with carbon number and unsaturations. This is exemplified by the ninth peak in Figure 1, which shows a comigration of linolenic acid methyl ester with eighteen carbons and three unsaturations, and arachidic acid methyl ester with twenty carbons and no unsaturations. Methyl linolenic acid actually has a slightly higher retention time as is seen in Table 1. The retention times of fatty acid methyl esters increase with carbon number and decrease with unsaturations when using the Tracor 560 capillary gas chromatograph. The seventh peak in Figure 2 is a comigration of oleic and linoleic acid methyl ester, methyl oleate, having fewer double bonds of the two, with a slightly higher retention time. The reason for this is that the packing of the Perkin Elmer packed column is polar, therefore, the more non-polar compounds elute earlier. The DB-1 column of the Tracor 560 is a non-polar column eluting polar compounds earlier. Utilization of both these column facilitates in confirmation of the identity of fatty acid methyl esters.

TABLE I

RETENTION TIMES (RT.) OF STANDARD FATTY ACID METHYL ESTERS

	Standards Injected Singly		Standards Injected Mixed	
Fatty Acid	Column A* Rt. (min.)	Column B** Rt. (min.)	Column A* Rt. (min.)	Column B** Rt. (min.)
C8	0.80	1.83	-	-
C10	1.15	2.45	1.08	2.38
C12	1.78	4.06	1.75	3.78
C14	3.15	6.02	3.10	5.93
C16	5.20	9.36	5.55	9.07
C16-1	6.25	8.06	6.55	8.69
C18	10.80	13.18	10.95	13.08
C18-1	11.90	13.05	11.70	12.44
C18-2	13.00	12.66	14.70	12.44
C18-3	19.40	12.64	19.70	12.35
C20	18.30	17.76	19.70	17.61
C20-4	34.43	16.02	35.90	15.89

*Column A represents data from a Perkin Elmer 990 packed column Gas Liquid Chromatograph. Column dimensions: 7.5' X 0.25 " o.d.; DEGS colunm packing; temperature: 180°C; flame ionization detector.

**Column B represents data from a Tracor 560 capillary column Gas Liquid Chromatograph. Column dimensions 30.0m. X 0.25mm.; DB-1 fused silica gel column; temperature: 75°C on column injection, increased to 180°C held for 4.0 min., raised 3° per min. until 230°C; flame ionization detector.

Figure 1. Chromatogram of Standard Fatty Acid Methyl Esters

Instrument: Perkin Elmer 990 Packed Column Gas Chromatograph. Column packing: Chromasorb W with 20% DEGS coating. Column dimensions: 7.5' x 0.25" Temperature: Isothermal 180°C. Detector Flame ionization.

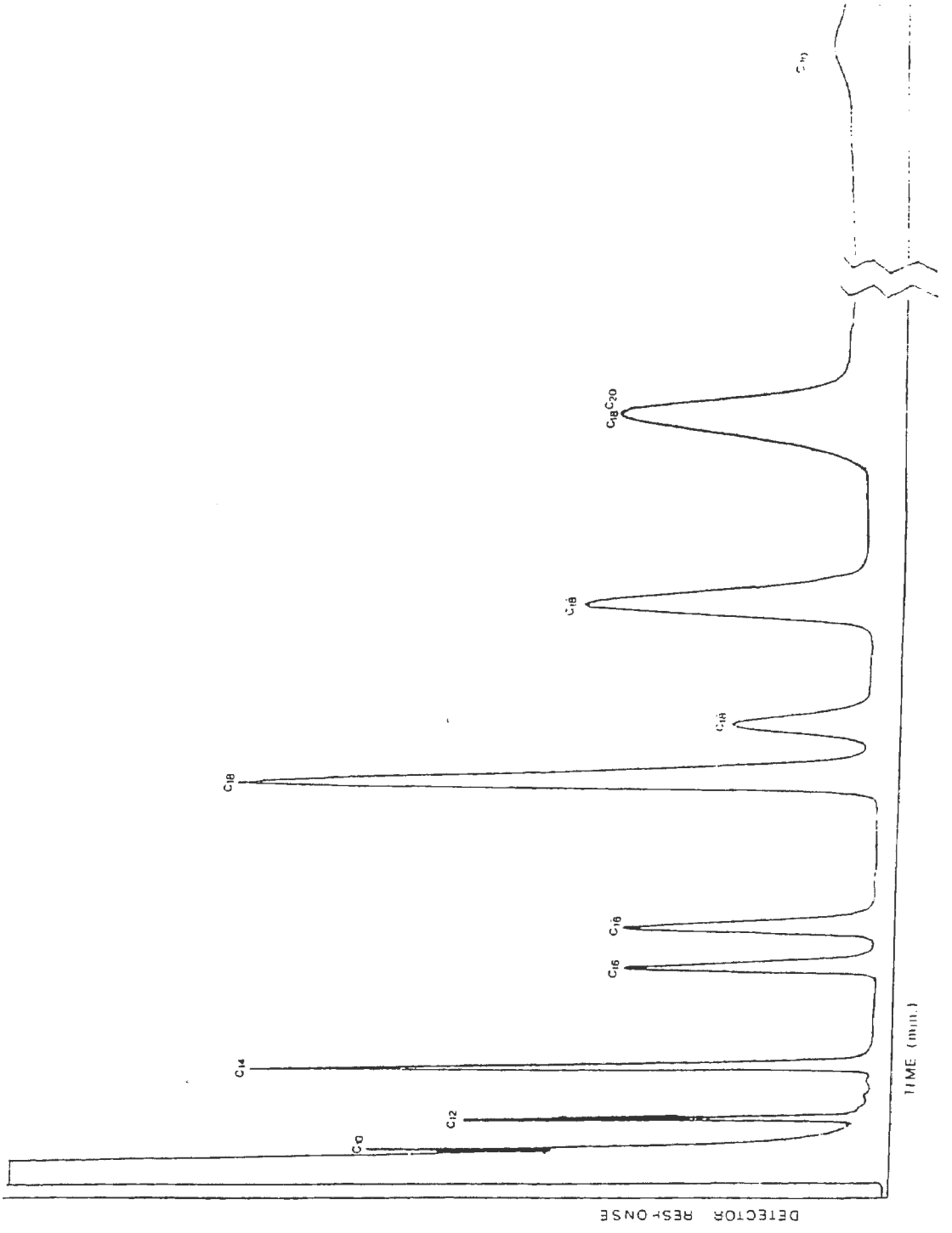
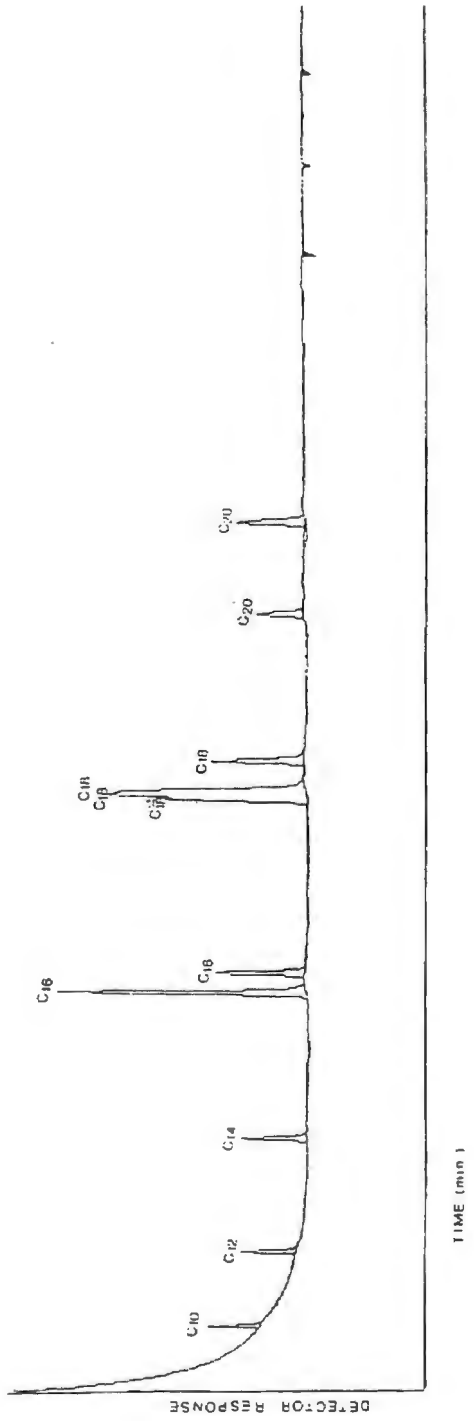

C8
C12
C14
C18
C16
C16
C18
C18
C18
C18C20
C22
DETECTOR RESPONSE
TIME (min.)

Figure 2. Chromatogram of Standard Fatty Acid Methyl Esters

Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica. Column dimensions: 3.0m x 0.25mm. Temperature programed for injection at 75°C,increased to 180°C held for 4.0 min., increased 3.0°/min. until 230°C. Detector: Flame ionization.


C10
C12
C14
C16
C18
C18
C18
C18
C20
C22
DETECTOR RESPONSE
TIME (min)

Identification by Gas Chromatography
of C. atrox Venom Lipids

C. atrox venom was transesterified prior to extraction of the lipids. Generally 20.0 mg of the dried whole venom was treated to transesterification resulting in a powdery precipate with an upper liquid layer containing the lipids. This lipid portion was injected into the gas chromatograph for identification of it's fatty acid content. Comparing the fatty acids observed in the venom using the Perkin Elmer 990 G. C. to the standard fatty acids methyl lauric acid was identified, as well as methyl myristic, methyl palmitic, methyl palmitoleic, methyl stearic, methyl oleic, methyl linoleic, methyl linolenic, and methyl arachidic acids. The solvent peak obscured the presence, if any, of methyl capric and methyl caprylic acids. After injection of the same lipid portion into the Tracor 560 capillary G. C. methyl laurate, methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate, methyl arachidonate were identified, based on standard fatty acids for that instrument. The possible presence of methyl caprate and methyl caprylate is again undeterminable due to the solvent peak. Chromatograms of the derivitized fatty acids found in C. atrox venom for both instruments are seen in Figure 3 and Figure 4.

A large peak is observed in the chromatogram of the packed column G. C. (Fig.3, peak 12) having a retention time of 35.0 minutes. This peak originally was tentatively identified as methyl arachidonate, however, after injection of a sample of the venom lipids spiked with a small amount of arachidonic acid methyl ester showed two distinct peaks, the peak was determined not to be arachidonic acid. Another possibility was that the peak represented behenic acid. A small amount of venom lipids was injected

Figure 3. Chromatogram of Standard Fatty Acids in *C. atrox* Venom Lipids

Instuments: Perkin Elmer 990 Packed Column Gas Chromatograph. column packing: Chromasorb W with 20° DEGS coating. Column dimensions: 7.5' x 0.25" Temperature: Isothermal 180°C. Detector: Flame ionization.

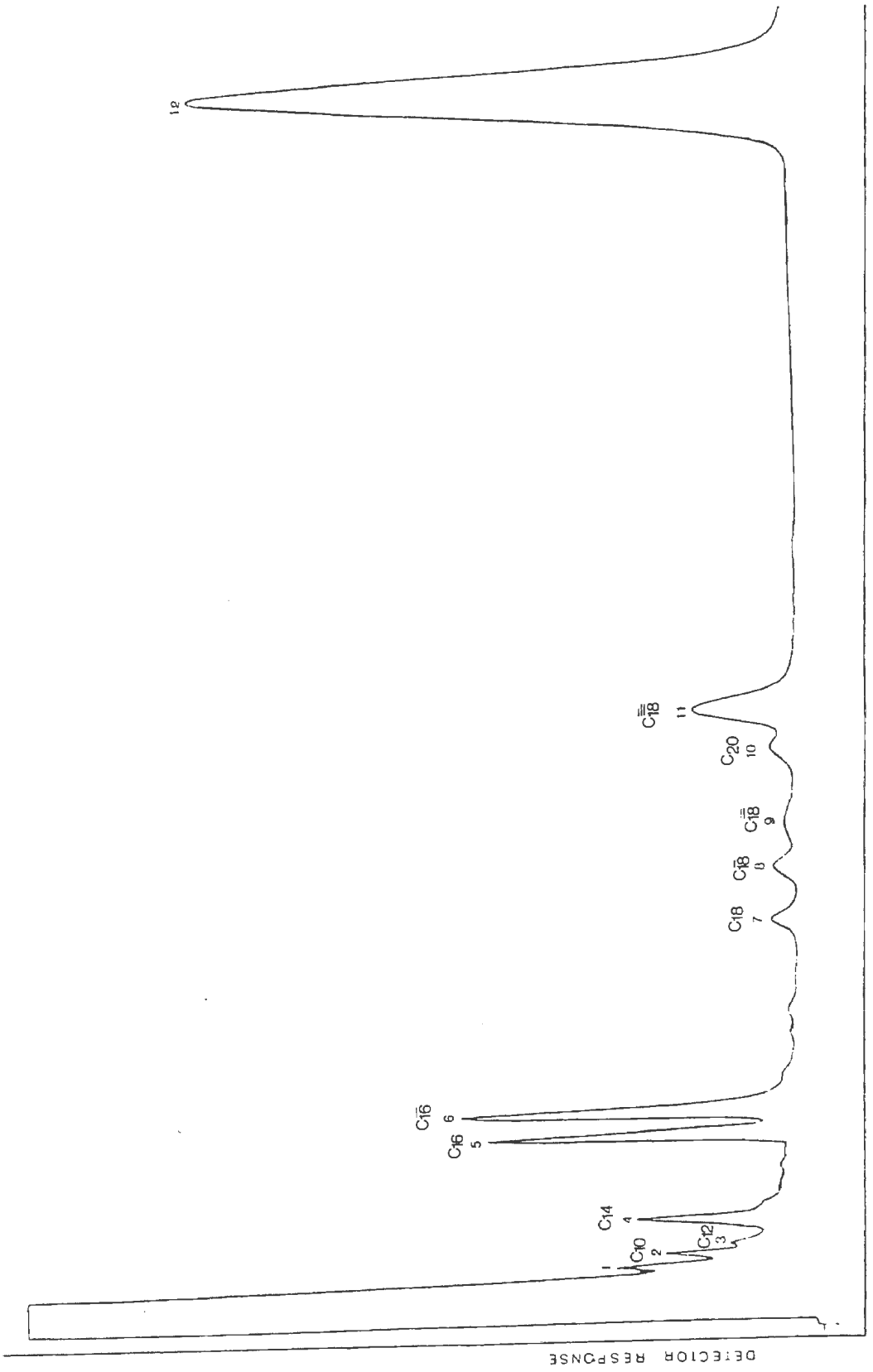
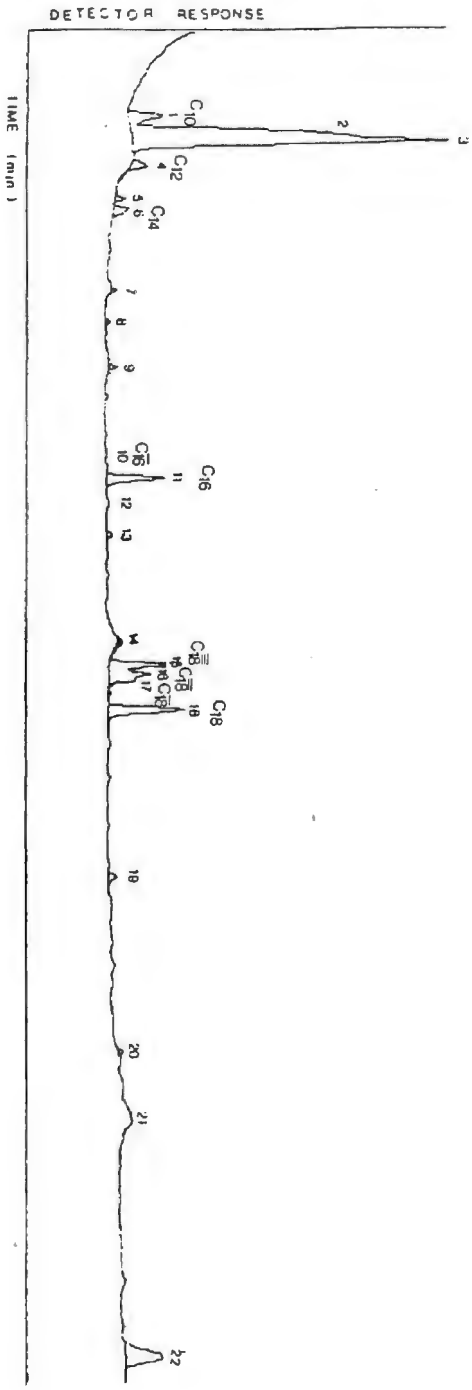

DETECTOR RESPONSE
TIME (min.)
1
C10 2
C12 3
C14 4
C16 5
C16 6
12
C18 11
C20 10
C18 9
C18 8
C18 7

Figure 4. Chromatogram os Standard Fatty Acids in *C. atrox* Venom Lipids

Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica. Column dimensions: 30.0m x 0.25mm. Temperature: Programed for injection at 75°C, increased to 180°C held for 4.0 min,, increased 3.0°/min. until 230°C. Detector: Flame ionization.


DETECTOR RESPONSE
TIME (min)
C10
C12
C14
C16
C18
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

with the addition of methyl behenic acid. Two peaks were again observed, therefore, the identity of the peak as behenic acid was rejected. That the unknown peak could not be methyl arachidonate or methyl behenate was further confirmed by the Tracor 560 chromatographic profile of the C. atrox venom lipids. The peak on this instrument has a retention time of three minutes, methyl arachidonate has a retention time of approximately fifteen minutes and methyl behenate twenty minutes. Based upon the retention times of the fatty acid standards available identification of the unknown peak by either instrument could not be accomplished. Further research found this peak did not appear if extraction of the lipids was performed prior to transesterification, indicating perhaps the peak is not a fatty acid or is bound to a non-extractable coumpound whose bonds are not broken by the transesterification procedure.

Quantitatively the most abundant venom fatty acids indentified by both instruments are palmitic, stearic and linoleic acids. Table II shows the relative percent of the fatty acids found in C. atrox venom.

Mass Spectrometry of C. atrox Venom Lipids

The LKB-2091 Gas Chromatograph/Mass Spectrometer was used for structural conformation of the fatty acids in C. atrox venom. Characteristic peaks for a fatty acid methyl ester according to Biochemical Applications of Mass Spectrometry (18) shows an ionization peak of 74 m/e the usual base peak for higher molecular weight methylated fatty acids. The ion fragment at 74 m/e represents the structure of $CH_3OCOHCH_2^{\bullet}$. Also a typical ionization peak for a fatty acid has a m/e of 143, the structure is $CH_3OCO(CH_2)_6^{\bullet}$. Standard fatty acid methyl esters were injected into the LKB-2091 and are contained in appendix A. Methyl stearate was confirmed to be present in

TABLE II

IDENTIFICATION, RETENTION TIMES AND RELATIVE PERCENT OF THE FATTY ACIDS IN C. ATROX VENOM

	Column A*			Column B**		
Fatty Acid	Peak No.	Rt. (min.)	Relative %	Peak No.	Rt. (min.)	Relative %
C8						
C10	2	1.85	1.76	1	2.65	13.22
C12	3	2.10	0.35	6	4.24	6.40
C14	4	2.75	7.96	8	6.87	2.69
C16	5	4.85	22.48	11	8.72	15.08
C16-1	6	5.50	29.27	10	-	1.65
C18	7	10.40	4.10	18	12.71	22.52
C18-1	8	11.40	3.86	17	12.12	2.27
C18-2	9	12.90	2.34	16	12.12	15.50
C18-3	11	15.70	22.13	15	11.97	16.53
C20	10	14.70	5.74	-	-	-
C20-4	-	-	-	19	15.49	4.13

*Column A represents data from a Perkin Elmer 990 packed column Gas Liquid Chromatograph. Column dimensions: 7.5 X 0.25"; DEGS column packing; temperature: 180°C; flame ionizaton detector

**Column B represents data from a Tracor 560 capillary column Gas Liquid Chromatograph. Column dimensions: 30.0m X 0.25mm; DB-1 fused silica gel column; temperature: 75°C injection, increased to 180°C held for 4 min., raised 3° per min. until 230°C; flame ionization detector.

the venom by it's ionization profile seen in Figure 5. The molecular ion 298 corresponding to the molecular weight of methyl stearate. Methyl palmitate was also confirmed by it's mass spectra seen in Figure 6. The molecular ion was 270 the molecular weight of palmitic acid methyl ester. Both ionization profiles exhibit the expected peaks at 74 m/e and 143 m/e. The peaks were subjected to the Wiley library search matching the spectra of the standard fatty acids to known fatty acid spectra.

The mass spectra profile of the large unknown peak, Figure 7, exhibited the fragmentation pattern of a fatty acid, however, the molecular ion was 185 which would not correspond to any common fatty acid methyl esters. That the peak was not behenic or arachidonic acid was confirmed.

Identification of the Fatty acids in the Lipid Classes of Snake Venom

The lipids of C. atrox venom were extracted into phospholipid, triglyceride, and sphingolipid fractions. One hundred and two hundred milligram samples were used. The triglycerides were first isolated by extraction using acetone, the phospholipids were extracted using a two to one chloroform/methanol solution, and the sphingolipids were isolated using hot ethanol.

A sample from each fraction was examined using thin layer chromatography. Standards from each lipid class mentioned above were spotted along with the samples. The phospholipids were the only class that was positively identified. Spots corresponding possibly to cholesterol were observed, however, a cholesterol standard was not spotted, therefore, this could not be confirmed.

The derivatized triglyceride, phospholipid, and sphingolipid fractions were injected into both the Perkin Elmer 990 packed column gas chromatograph and the Tracor 560 capillary column gas chromatograph. The chroma-

Figure 5. Mass Spectra of Stearic Acid Methyl Ester Observed in C. atrox Venom Lipids

Instrument: LKB-2091 Capillary GC/MS.
Column: DB-5 fused silica. Column Dimensions: 60m x 0.25mm with 1.0 microliter film;
Temperature: Injected at 100°C, increased 10° per min. until 280°C held for 15 min.; flushed with hexane.

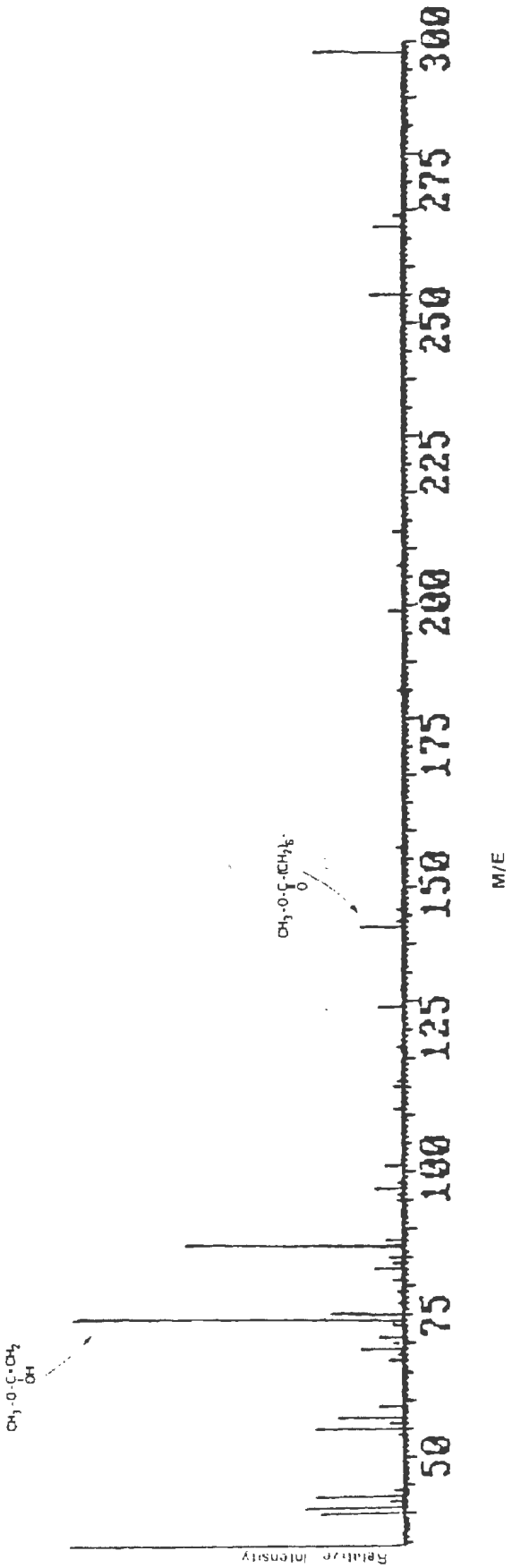

Relative Intensity
50
75
100
125
150
175
200
225
250
275
300
M/E
CH3-O-C-CH2
OH
CH3-O-C-(CH2)6
O

Figure 6. Mass Spectra of Palmitic Acid Methyl Ester Observed in C. atrox Venom Lipids

Instrument: LKB-2091 Capillary GC/MS
Column: DB-5 fused silica. Column Dimensions: 60m x 0.25mm with 1.0 microliter film;
Temperature: Injected at 100°C, increased 10° per min. until 280°C held for 15 min.; flushed with hexane.

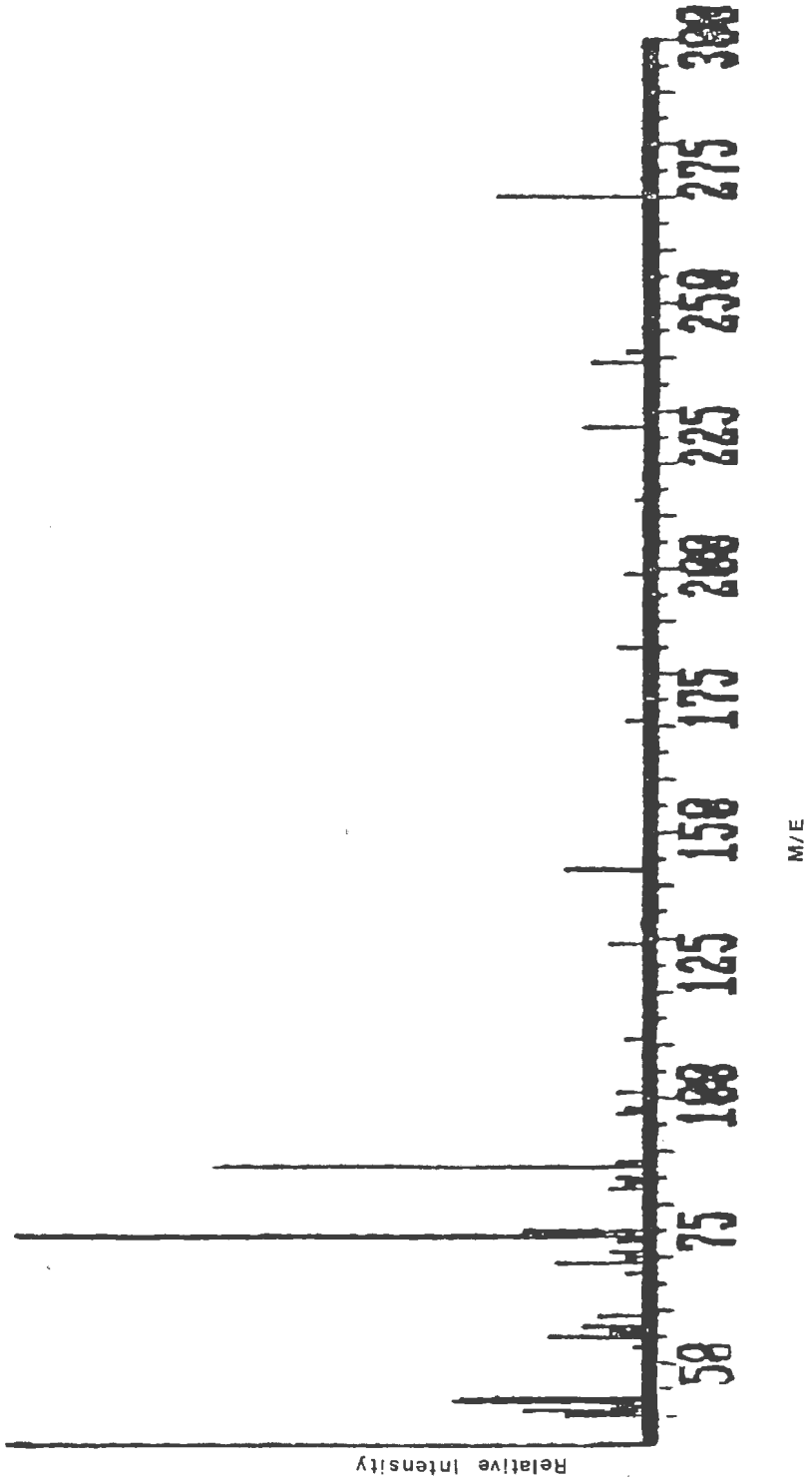
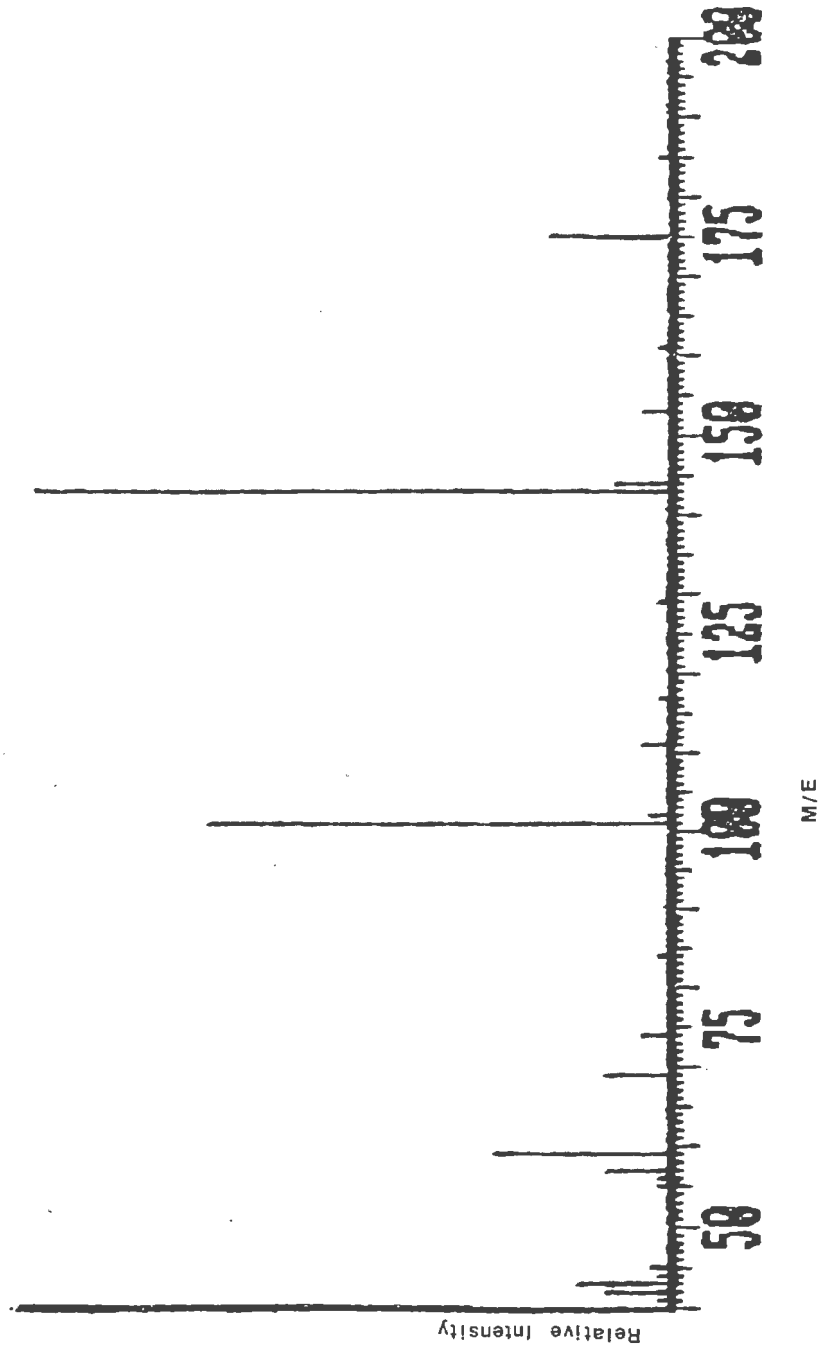

Relative Intensity
58
75
100
125
150
175
200
225
250
275
300
M/E

Figure 7. Mass Spectra of Unknown Peak Observed in *C. atrox* Venom Lipids

Instrument: LKB-2091 Capillary GC/MS.
Column: DB-5 fused silica. Column Dimensions: 60m x 0.25mm with 1.0 microliter film;
Temperature: Injected at 100°C, increased 10° per min. until 280° held for 15 min.; flushed with hexane


Relative Intensity
50
75
100
125
150
175
200
M/E

tograms of the derivitized fatty acids can be seen in Figures 8-13. The large unidentified peak is not observed in any of the extracted lipid fractions. The packed column Perkin Elmer G. C. identified capric, lauric, palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acid methyl ester. The capillary column Tracor 560 G. C. also indentified and confirmed identification of these fatty acid methyl esters as well as arachidic and arachidonic acid methyl esters. The fatty acids in the phospholipid fraction identified by the packed column G. C. were myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acid methyl esters. The capillary column G. C. confirmed the identity of palmitic, palmitoleic, stearic, oleic, and linolenic acid methyl ester. The sphingolipid component of the venom according to the packed column G. C. contains capric, lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidic acid methyl esters. The capillary column confirmed capric, myristic, palmitic, stearic, oleic, linolenic, and arachidic acid methyl esters in the sphingolipid fraction and also arachidonic acid methyl ester. Linoleic acid methyl ester may have been present but was indistinguishable from oleic acid.

A table of the fatty acids in the C. atrox venom lipid classes and their relative percentages can be seen in Table III.

Comparision of the Fatty Acids in the Liver, Adipose Tissue, Venom Gland, and Venom of C. viridis viridis

A comparision of the fatty acids in the lipids of the venom, venom gland, adipose tissue and liver of C. viridis viridis was conducted. Adipose tissue, venom glnads and liver were removed from four snakes, two of which had recently been fed while the other two had not. The tissues were ex-

Figure 8. Chromatogram of the Fatty Acids in the Triglyceride Fraction of *C. atrox* Venom Lipids

Instrument: Perkin Elmer 990 Packed Column Gas Chromatograph. Column packing: Chromasorb W with 20% DEGS coating. Column dimensions: 7.5' x 0.25". Temperature: Isothermal 180°C. Detector: Flame ionization.

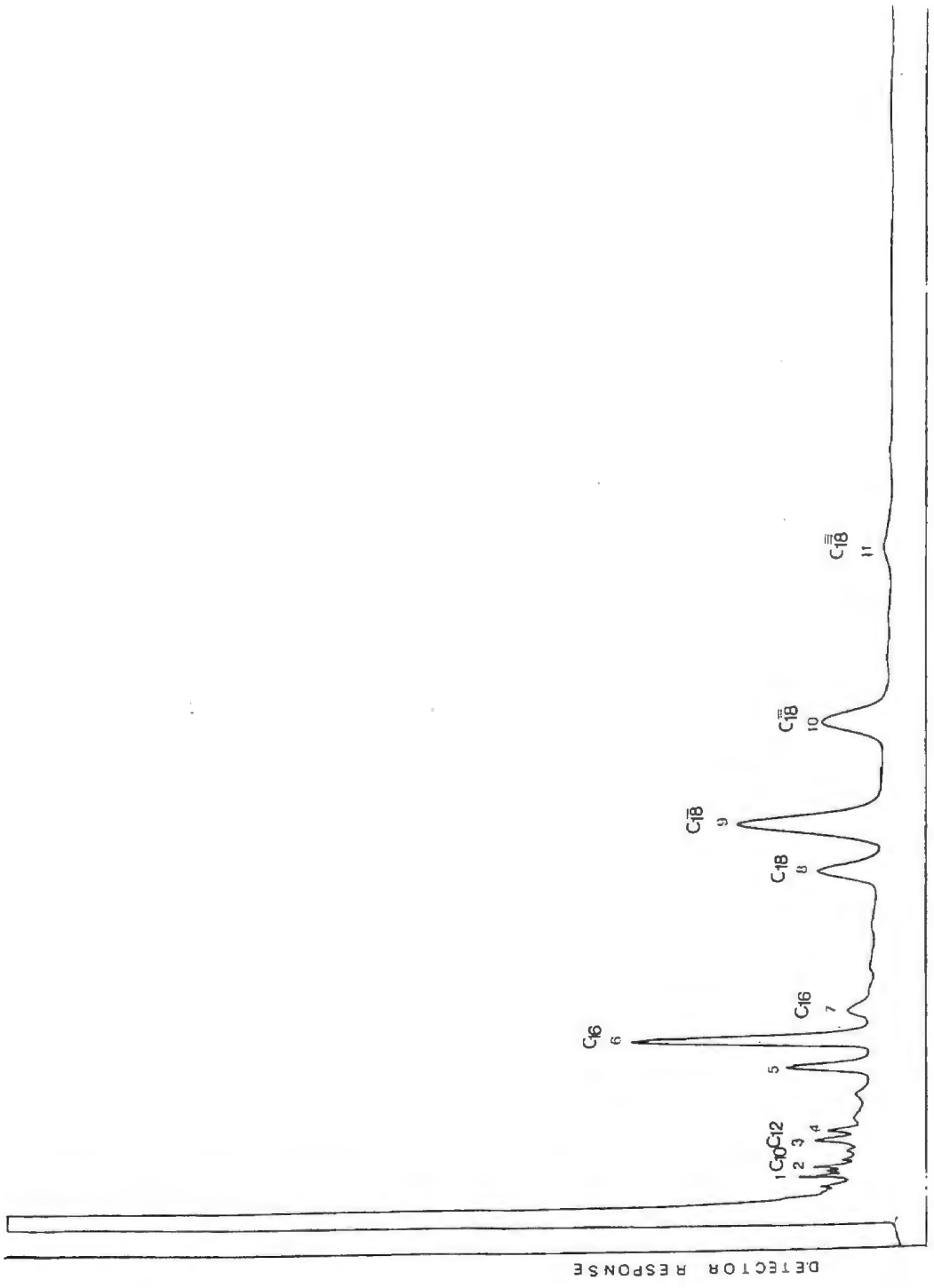

DETECTOR RESPONSE
TIME (min)
C10
C12
C16
C16
C18
C18
C18
C18
1
2
3
4
5
6
7
8
9
10
11

Figure 9. Chromatogram of the Fatty Acids in the Triglyceride Fraction of *C. atrox* Venom Lipids

Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica. Column dimensions: 30.0m x 0.25mm. Temperature Programed for injection at 75°C, increased to 180°C held for 4.0 min., increased 3.0°/min. until 230°C. Detector: Flame ionization.

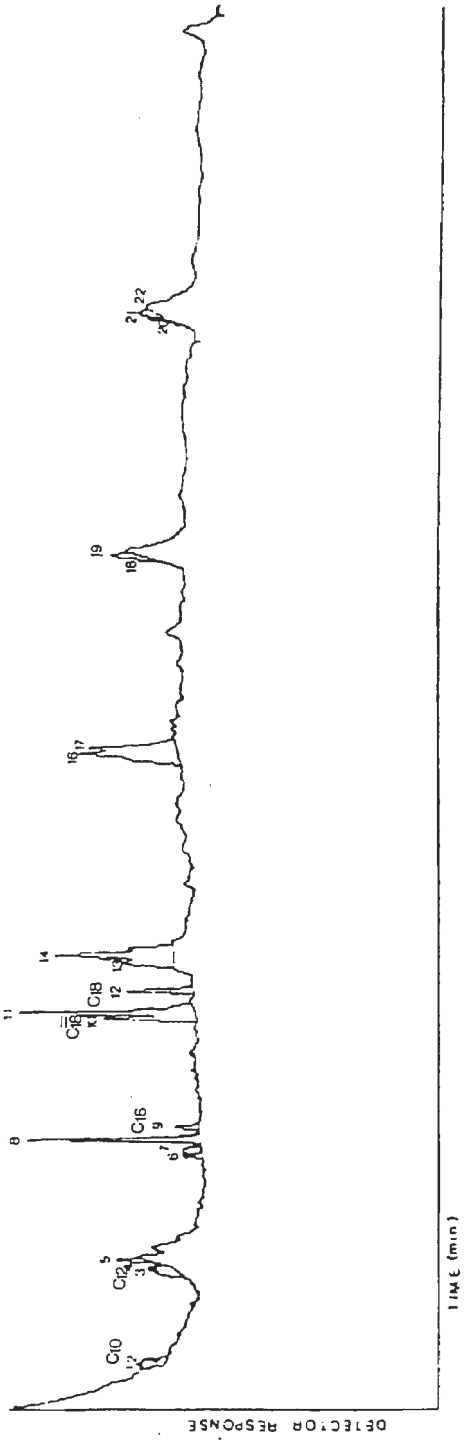

DETECTOR RESPONSE
TIME (min)
C10
C12
C16
C18
C18
C16
C18
C16
1
2
3
4
5
6
7
8
9
10
11
12
13
14
16
17
18
19
21
22
23

Figure 10. Chromatogram of the Fatty Acids in the Phospholipid Fraction of *C. atrox* Venom Lipids

Instrument: Perkin Elmer 990 Packed Column Gas Chromatograph. Column packing: Chromasorb W with 20% DEGS coating. Column dimensions: 7.5'x 0.25". Temperature: Isothermal 180°C. Detector: Flame ionization.

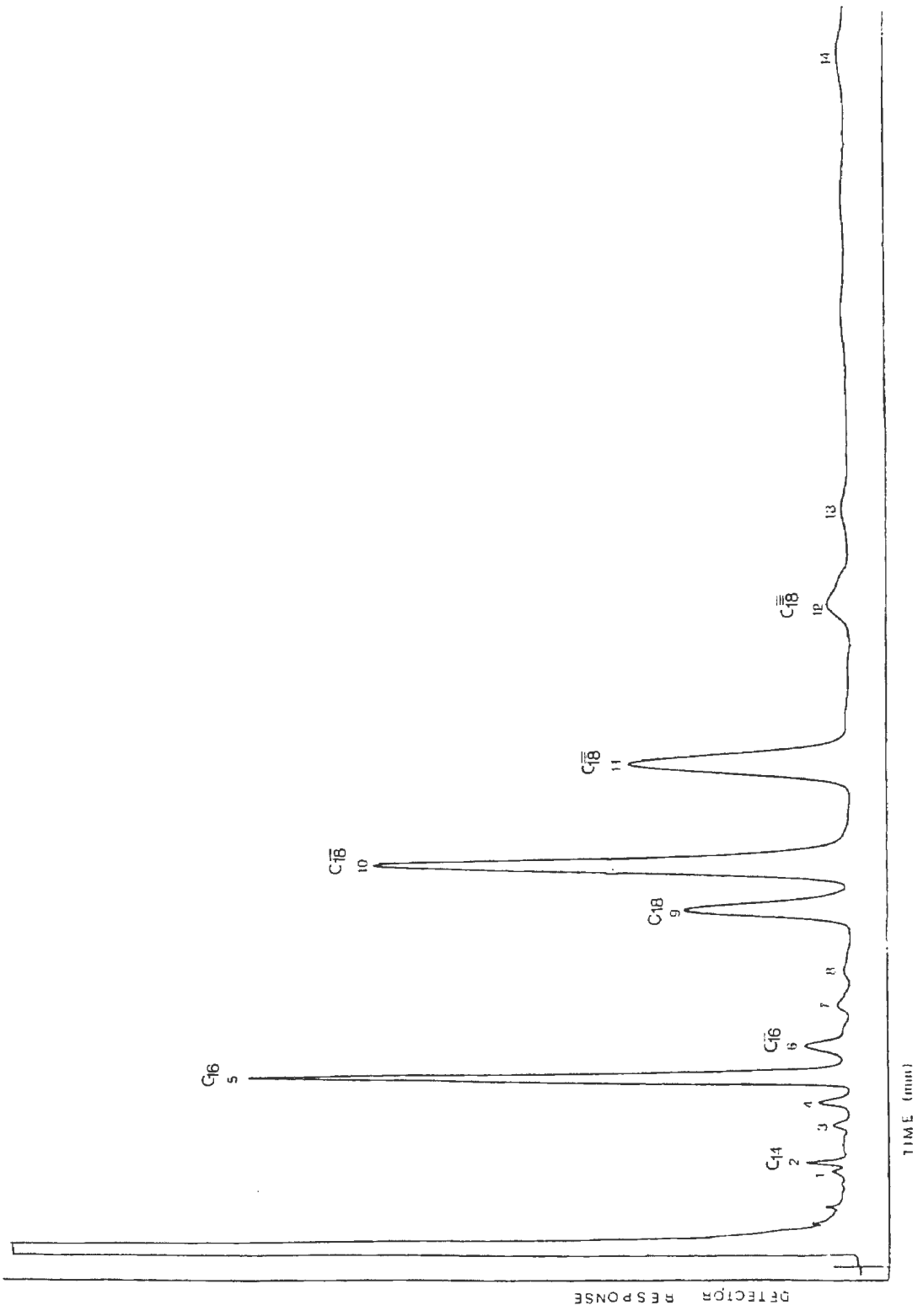

DETECTOR RESPONSE
TIME (min)
C14
2
C16
5
C16
6
C18
9
C18
10
C18
11
C18
12
13
14

Figure 11. Chromatogram of the Fatty Acids in the Phospholipid Fraction of *C. atrox* Venom Lipids

Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica. Column dimensions: 30.0m x 0.25mm. Temperature Programed for injection at 75°C, increased to 180°C held for 4.0 min. increased 3.0°/min. until 230°C. Detector: Flame ionization.

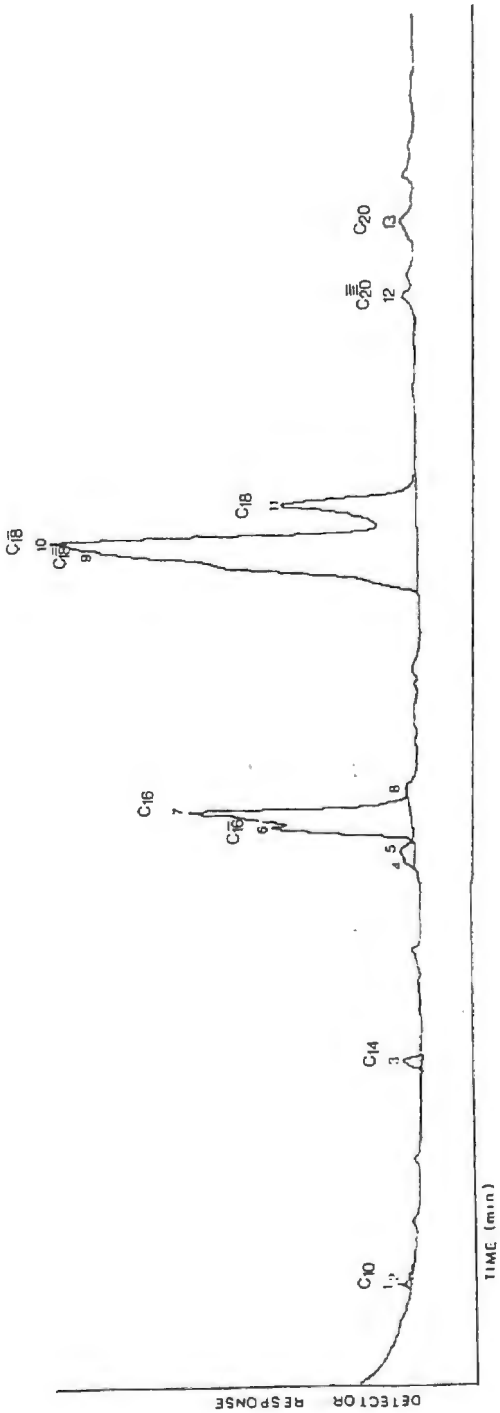

DETECTOR RESPONSE
TIME (min)
C10
1
2
C14
3
4,5
C16
7
C16
6
8
C18
11
C18
10
C18
9
C20
12
C20
13

Figure 12. Chromatogram of the Fatty Acids in the Sphingolipid Fraction of C. atrox Venom Lipids

Instrument: Perkin Elmer 990 Packed Column Gas Chromatograph. Column packing: Chromasorb W with 20% DEGS coating. Column dimensions: 7.5' x 0.25". Temperature: Isothermal 180°C. Detector: Flame ionization.

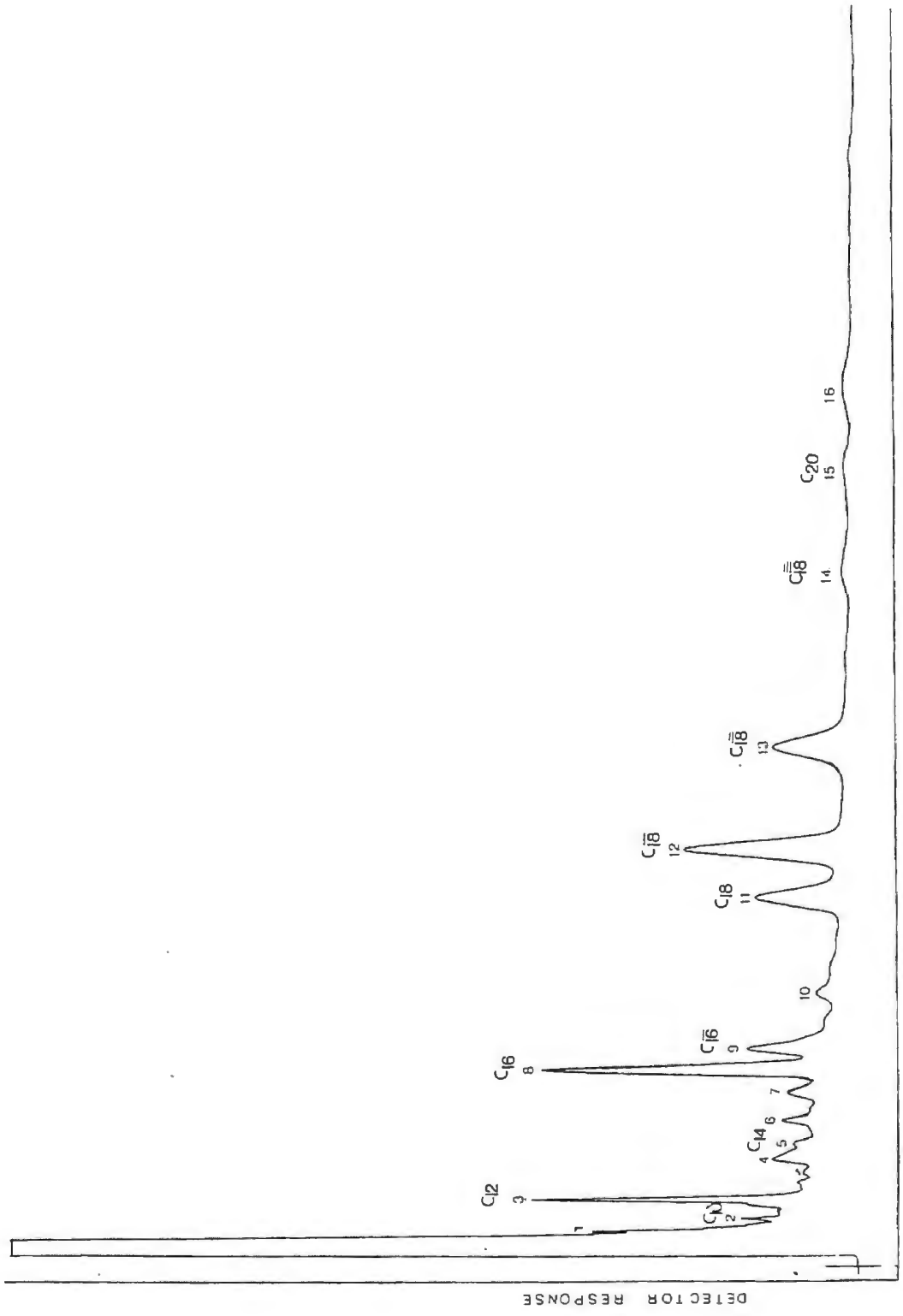

DETECTOR RESPONSE
TIME (min)
C12
C14
C16
C16̄
C18
C18̄
C18̄
C18̄
C20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 13. Chromatogram of the Fatty Acids in the Sphingolipid Fraction of C. atrox Venom Lipids

Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica. Column dimensions: 30.0m x 0.25mm. Temperature: Programed for injection at 75°C, increased to 180°C held for 4.0 min., increased 3.0°/min. until 230°C. Detector; Flame ionization.

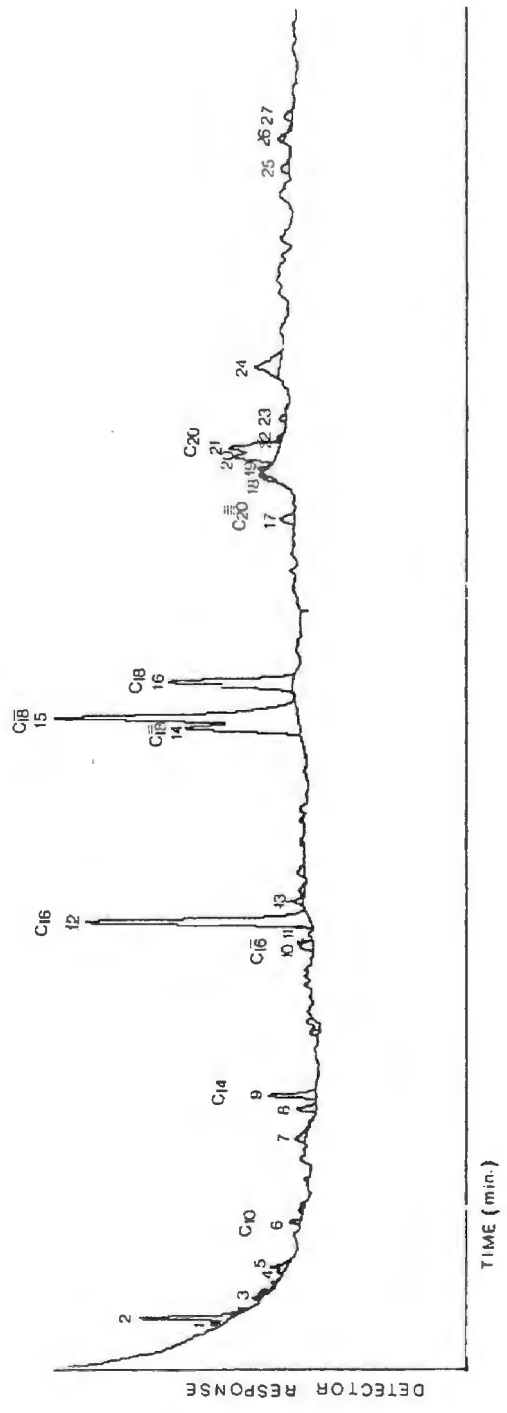

DETECTOR RESPONSE
TIME (min.)
C14
C16
C16=
C18
C18=
C18==
C20
C20=
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27

TABLE III

Identification, and Relative Percent of the Fatty Acids in the Triglyceride, Phospholipid, and Sphingolipid Fractions of *C. Atrox* Venom

	Triglyceride Fraction						Phospholipid Fraction						Sphingolipid Fraction					
	Column A*			Column B**			Column A*			Column B**			Column A*			Column B**		
Fatty Acid	Peak No.	Rt. (min.)	Relative %	Peak No.	Rt. (min.)	Relative %	Peak No.	Rt. (min.)	Relative %	Peak No.	Rt. (min.)	Relative %	Peak No.	Rt. (min.)	Relative %	Peak No.	Rt. (min.)	Relative %
C8	-	-	-	-	-	-												
C10	2	1.75	1.86	2	2.51	6.34	-	-	-	1	2.60	0.55	2	1.20	1.13	6	2.87	1.72
C12	3	2.40	2.33	3	4.96	7.20	-	-	-	-	-	-	3	1.75	9.22	-	-	-
C14	-	-	-	-	-	-	2	2.75	0.67	3	5.57	1.02	5	3.10	1.42	9	5.88	4.37
C16	6	4.85	27.04	9	8.84	6.05	5	5.00	20.52	7	8.86	15.84	8	5.10	24.40	12	8.73	22.41
C16-1	7	5.50	3.50	8	8.50	21.90	6	5.80	1.33	6	8.67	6.01	9	5.60	4.26	10	8.29	2.53
C18	8	9.00	13.05	12	12.48	12.10	9	9.40	11.46	11	12.98	13.80	11	9.40	11.35	16	12.74	14.02
C18-1	9	10.10	28.44	11	11.91	31.12	10	10.65	40.31	10	12.43	29.92	12	10.60	27.66	15	12.17	32.18
C18-2	10	12.60	20.51	10	11.73	15.27	11	13.20	22.38	9	12.33	31.39	13	13.05	12.77	14	11.99	13.68
C18-3	11	16.90	3.26	-	-	-	12	17.25	3.31	-	-	-	14	17.40	2.84	-	-	-
C20	-	-	-	-	-	-	-	-	-	13	16.02	0.64	15	20.20	4.96	21	16.75	6.44
C20-4	-	-	-	-	-	-	-	-	-	12	17.39	0.83	-	-	-	17	15.53	2.64

*Column A represents data from a Perkin Elmer 990 packed column Gas Liquid Chromatograph. Column dimensions: 7.5' X 0.25"; DEGS column packing; Temperature: 180°C, flame ionization detector.

**Column B represents data from a Tracor 560 capillary column Gas Liquid Chromatograph. Column dimensions: 30.0m X 0.25mm; DB-1 fused silica gel column; temperature: 75°C injection, increased to 180°C held for 4 min., raised 3° per min. until 230°C; flame ionization detector.

tracted into triglyceride, phospholipid, and sphingolipid lipid fractions. The fatty acids of the lipid fractions were determined by capillary column gas chromatography. The lipids of the venom of a different C. viridis viridis snake was also examined for it's triglyceride, phospholipid, and sphingolipid fatty acid content by capillary column gas chromatography. The results of this analysis can be seen in Table IV. No significant difference in the relative percent of the fatty acids was observed between the tissue from the snakes that were fed and those which were not.

All three classes of lipid in the venom gland contained most all of the fatty acids for which standards were available. The adipose tissue contained the higher molecular weight fatty acids in the triglyceride and sphingolipid classes with the exception of methyl arachidate and arachidonate which were observed in the phospholipid fraction. All three lipid fractions of the liver and venom lipids contained the higher molecular fatty acids. The most abundant fatty acids observed in all samples were stearic and palmitic acids.

The Fatty Acid Content of the Venom Lipids of Eight Different Species or Sub-Species of Snake

A comparision of the fatty acids in the lipid component of venom from eight different species or sub-species of snakes was conducted. All eight snakes were of the crotalidae family including four rattlesnakes: C. adamanteus, easter diamond back rattlesnake, C. h. horridus, timber rattlesnake, and Sistrurus miliarius barbouri, pigmy rattlesnake; three venoms from the Agkistrodon genus: A. contortrix contortrix, southern copperhead. A. contortrix mokasen, northern copperhead, and A. piscivorus piscivorus, cottonmouth, and Bothrops asper, Central American viper.

TABLE IV

*Identification and Relative Percent of Fatty Acids in the Liver, Adipose Tissue, Venom Gland, and Venom of *C. viridis viridis*

	Venom Lipids			Venom Glands			Adipose Tissue			Liver		
Fatty Acid	TG** Relative %	PS*** Relative %	Sp**** Relative %	TG Relative %	PS Relative %	SP Relative %	TG Relative %	PS Relative %	SP Relative %	TG Relative %	PS Relative %	SP Relative %
C8	-	-	-	-	-	-	-	-	-	-	-	-
C10	-	-	-	0.42	1.51	3.72	-	-	-	-	-	-
C12	-	-	-	0.36	-	0.46	-	2.15	-	0.15	-	-
C14	4.59	-	-	0.69	6.81	2.70	-	3.10	-	0.22	0.38	-
C16	30.51	34.56	65.46	24.70	31.31	23.11	25.22	29.46	29.20	16.89	21.36	28.36
C16-1	5.64	22.12	15.94	0.86	15.66	6.48	2.88	2.55	-	3.24	1.0	-
C18	20.63	19.35	13.77	10.40	32.28	29.05	20.80	38.92	43.36	10.56	18.88	23.57
C18-1	18.53	17.51	4.83	18.52	13.00	111.66	38.50	24.12	13.27	57.97	31.14	33.99
C18-2	17.64	6.45	-	32.64	9.68	14.74	-	15.02	14.14	-	25.30	23.34
C18-3	-	-	-	13.02	7.65	8.10	-	-	-	-	-	-
C20	-	-	-	0.36	1.14	-	-	3.28	-	0.50	1.75	4.45
C20-4	2.47	-	-	9.42	6.47	6.61	-	1.88	-	10.68	9.00	19.48

*Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica, column dimensions: 30.m x 0.25mm. Temperature; Programed for Infection at 75°C, increased to 180°C held for 4.0 mnl., increased 3.0°/min. until 230°C. Detector : Flame Ionization.

**Triglyceride Fraction

***Phospholipid Fraction

****Sphingolipid Fraction

The lipids were extracted from the venoms with ether, transesterified and analyzed by capillary G. C. for fatty acid content. Whole venom samples were also transesterified and the upper lipid containing layer analyzed for fatty acids. Table V shows a comparative table of the fatty acids in the venoms of the snakes and their relative percent.

The chromatogram of the methylated fatty acids in the whole transesterified venom exhibited the previously mentioned unidentifed peak (peak 12), whereas, the chromatogram of the fatty acids in which the lipids were first extracted with ether before methylation this peak was absent. Figures 14a and 14b are the chromatograms of the whole transesterified venom methylated fatty acids and the venom fatty acids extracted with venom before. transesterification of Sistrurus miliarius barbouri. This figure is shown as a representative illustration of the different chromatographic profiles of the fatty acids in whole venom after transesterification and fatty acids in which the lipids are extracted before transesterified. Figure 14a is the whole venom transesterified fatty acid methyl esters with the unidentified peak at 3.0 minutes (peak 12) being the predominate feature. As table V indicates few other fatty acids are present in significant amounts. Figure 14b contains the fatty acids is due to the absence of the large peak seen in transesterified whole venom which causes a reduction in observable fatty acids. The chromatograms of the other seven snake venom fatty acids are contained in appendix B.

Figure 14. Chromatogram A. Fatty Acid Methyl Esters in the Whole Venom of Sistrurus m. borbouri. Chromatogram B.Fatty Acid Methyl Esters in the Extracted Venom Lipids of Sistrurus m. barbouri

Instrument: Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica. Column dimensions: 30.0m x 0.25mm. Temperature: Programed for injection at 75°C, increased to 180°C held for 4.0 min., increased 3.0°/min. until 230°C. Detector: Flame ionization.

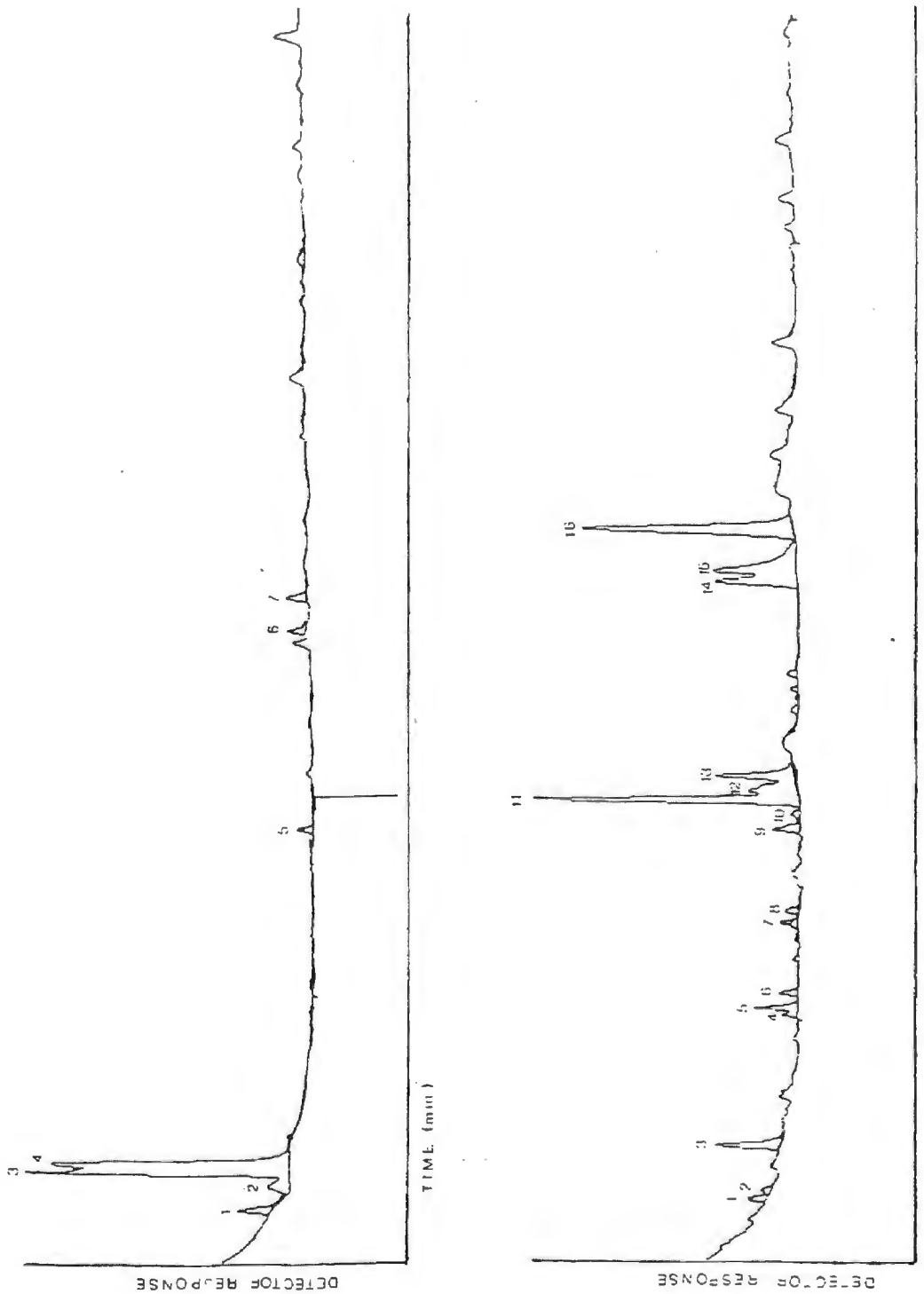

DETECTOR RESPONSE
TIME (min)
1
2
3
4
5
6
DETECTOR RESPONSE
TIME (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

TABLE V

*Comparision of Relative Percent of the Fatty Acids in the Venom Lipids from Eight Different Species or Sub-Species of Snakes

	Crotalus atrox		Crotalus adamantues		Crotalus h. horridus		Agkistrodon c. mokasen		Agkistrodon p. piscorvorus		Sistrurus m. barabouri		Agkistrodon c. contortrix		Bothrops asper	
	Relative %		Relative %		Relative %		Relative %		Relative %		Relative %		Relative %		Relative %	
Fatty Acid	1**	2***	1	2	1	2	1	2	1	2	1	2	1	2	1	2
C8	-	-	-	-	-	-	-	-	-	2.65	-	-	-	-	-	-
C10	13.22	-	40.00	-	13.01	3.62	-	-	10.05	5.71	39.69	2.15	25.13	-	21.25	-
C12	6.40	6.89	17.14	-	9.59	3.62	-	4.19	14.67	5.29	-	7.42	5.35		15.00	-
C14	2.69	6.32	-	-	-	5.25	3.74	4.76	-	2.78	-	2.36	-	8.11	6.25	5.78
C16	15.08	8.42	9.71	43.64	8.90	4.17	-	12.19	15.76	10.72	16.03	8.82	13.90	11.71	11.88	14.63
C161	1.65	20.00	-	-	-	33.88	-	23.81	-	25.49	-	23.98	-	23.42	-	34.01
C18	22.52	24.74	10.86	56.36	21.23	36.23	5.14	28.95	25.54	31.75	22.90	29.14	23.00	27.93	11.25	36.05
C181	2.27	7.37	9.71	-	11.64	8.88	7.94	2.86	26.90	9.75	21.37	14.73	18.18	-	10.62	-
C182	15.50	18.42	12.57	-	13.70	4.35	11.68	12.00	7.06	3.70	-	11.40	7.83	-	-	-
C183	16.53	-	-	-	-	-	-	6.29	-	2.65	-	-	-	-	-	-
C20	-	4.21	-	-	12.33	-	29.44	3.05	-	-	-	-	4.81	28.83	-	-
C204	4.13	3.68	-	-	9.59	-	42.06	1.90	-	-	-	-	-	-	23.75	9.52

*Instrument : Tracor 560 Capillary Column Gas Chromatograph. Column: DB-1 fused silica, column dimensions: 30.0m x 0.25mm. Temperature: Programed for injection at 75°C, increased to 180°C held for 4.0 min., increased 3.0°C/min. until 230°C. Detector: Flame ionization.

**Column 1 represents the fatty acid methyl esters of transesterified whole venom.

***Column 2 represents the fatty acid methyl esters of the transesterified venom lipids after lipid extraction with diethyl ether.

CHAPTER V

SUMMARY

The role of lipids in the venom of snakes, though not completely understood, is known to be non-passive, It has been demonstrated that lipids have an enhancing effect on the toxicity of venom (4,5,6,16). The purpose of this research has been to characterize the lipids found in venom. the venom of C. atrox has been volatilized by transesterification techniques and the methylated fatty acids determined by packed column gas chromatography, capillary column gas chromatography, and gas chromatography/mass spectrometry. The following fatty acids were identified in C. atrox by packed column gas chromatography and confirmed by capillary gas chromatography and are listed in order of abundance: Linolenate, palmitate, stearate, myristate, oleate, linoleate, caprate, and laurate. Stearate and palmitate were confirmed by GC/MS.

Identification of the fatty acids in the lipid classes of snake venom was accomplished by lipid extraction of the triglyceride, phospholipid, and sphingolipid followed by analysis of fatty acids using packed and capillary column gas chromatography. Stearate, oleate, linoleate, palmitate, and palmitoleate, were the most abundant fatty acids identified by both instuments. The triglyceride represents the neutral lipids extracted by acetone prior to the extraction of the phospholipids with ether. The phospholipids were confirmed by thin layer chromatography and appeared to be the predominate lipid present. The sphingolipid fraction contains the residual lipids

extracted with hot ethanol after the ether extraction. The sphingolipids may contain lipids other than sphingolipids as no sphingosine determination was conducted. A comparison of the fatty acids in the classes of lipids in the venom lipid, venom gland, adipose tissue, and liver of C. viridis viridis shows the presence of stearate, oleate, linoleate, and palmitate, of the venom lipids to be of the same relative amount as observed in the tissue samples. However, while the venom gland, liver, and phospholipids of the adipose tissue contain arachidic and arachidonic acids the venom lipids contained no significant amount of twenty carbon fatty acids.

In the comparision of the fatty acids in the venom of C. adamanteus, C. atrox, B. asper, A. c. contortrix, S. m. barbouri, A. p. piscivorus, C. h. horridus, and A. c. mokasen, stearate was the only fatty acids present in all snake venom. Palmitate was present in all venoms with the exception of A. c. mokasen. Agkistrodon c. mokasen and Bothrops asper were the only two snakes who's venom contained significant quantities of arachidonic acid.

In summary the findings of this study shows the presence of predominatly higher molecular fatty acids containing sixteen or eighteen carbons, saturated and a considerable amount of unsaturated fatty acids, specifically oleic acid. This is consistent with the fatty acids observed in other snake tissue with the exception of the absence of arachidic and arachidonic acids. The lack of arachadonic acid is a notable observation, as the findings of Kabarra and Fischer (4) identified arachidonate as the most abundant fatty acid in Naja naja venom. In our research the lipids of Naja naja venom were analyzed on both packed and capillary column gas chromatographs and no arachidonic acid was observed. This led to the speculation that since Kabarra and Fischer were basing their indentification onthe retention time of a packed column G. C. the peak they observed was the same we observed

earlier in our work when we transesterified the venom before lipid extraction and a very large peak (70% of total lipid) with the approximate retention time of arachidonic acid was present. The retention time of this peak on a non-polar capillary column was at a considerably earlier time than standard arachidonic acid causing the initial rejection of arachiconic acid as this peak. This peak was not observed when extraction of the lipids from the venom was done prior to transesterification. The mass spectra of this peak possesses characteristics of a fatty acid suggesting a lipid attached molecule whose bonds are not brolen by the transesterification process but is not extractable into the lipid fraction.

The fatty acids in different snake venom lipids were similar, containing primarily palmitic and stearic acids. All of the venoms were desicated venoms which had been stored for varying amounts of time. Although research has been conducted on the activity of venoms stored over time and little change in activity was indicated, (18) the fatty acids may undergo some alterations. Future studies involving lipids should investigate this possibility. Other considerations for future research on lipids include assays for biological activity of the lipid fractions of venom as well as the total lipid.

BIBLIOGRAPHY

1. Russell, F. E., Experientia, 30, 8, (1974).

2. Habermaehl, G. G., Venomous Animals and Their Toxins, Springer- Verlag, New York, (1977).

3. Tu, A. T., Venom: Chemistry and Molecular Biology, John Wiley and Sons, Inc., New York, (1977).

4. Kabarra, j. j., and Fischer, G. H., Toxicon, 7, 223, (1969).

5. Moroz, C., De Vries, A., and Sela, M., Boichemica et Biophysica Acta, 124, 136, (1966).

6. Bon, C., and Radvanyl, F., "Investigations on Crotoxin Mechanism of Action", American Chapter of the International Society of Toxinology, Stillwater, Oklahoma, (1982).

7. Gangulay, S. N., and Malkana, M. T., Indian Journal of Medical Research, 24, 281, (1936).

8. Bucherl, W., Buckley, E. E., and Deulofeu, V., Venamous Animals and Their Venoms, Academic Press, Inc., New York, 119-165, (1968).

9. Buckley, E. E., and Porges, N., Venoms, American Association of the Advancement of Science, New York, 153-169, (1956).

10. Grasset, E., and Schwartz, D. E., Ann. Inst. Pasteur, 88,271-81 (1955).

11. Devi A., Unpublished Observations, (1968), Venamous Animals and Their Venoms, Academic Press, Inc., New York.

12. McFarlane R. G., Trevan, J. W., and Attwood, A.M.P., Journal of Physiol (london) 99,7P.

13. Fault, P., and Everard, B. A., Australian J. Expt. Biol. Med. Sci., 27,197, (1949).

14. Poole, J. C. F., Robinson, D. S., and MacFarlane, R. G., Quart. J. Exptl. Physiol.40, 276, (1941).

15. Poole, J. C. F., and Robinson, D. S., Quart. J. Exptl. Physiol., 41, 295,(1956).

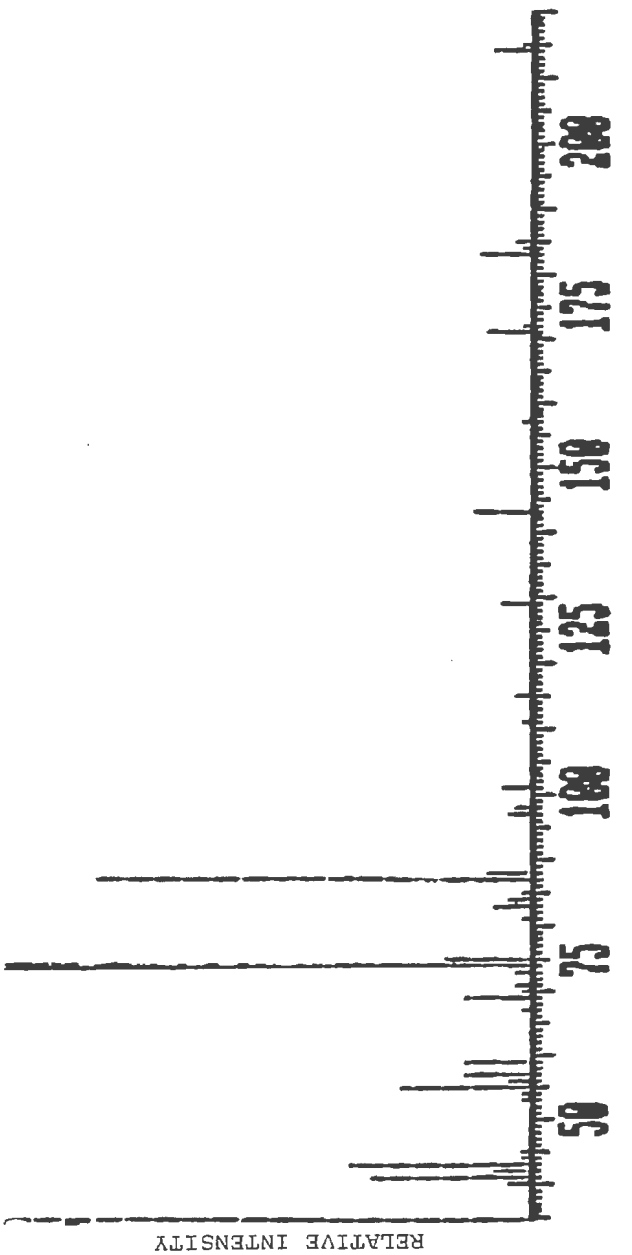
16. Bon, C., Radvanyl, F., Faure, G., and Saliou, B., "Crotoxin Structure Function and Mode of Action." American Chemical Society, 5th Rocky Mountain Regional Meeting, June, 1986.

17. Mason, M. E., and Waller, G. R., Anal. Che., 36, 583, (1964).

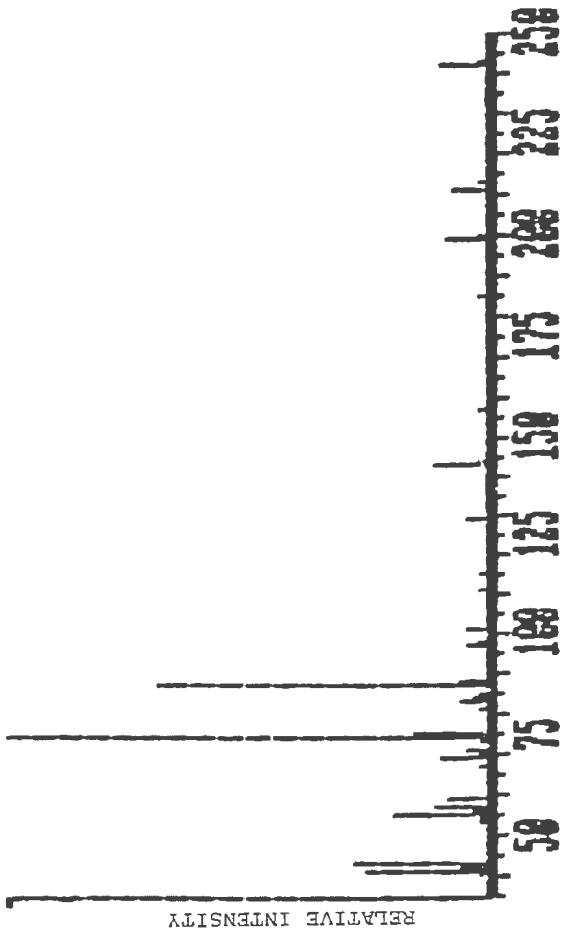
18. Waller, G. P., Biochemical Applications of Mass Spectrometry, John Wiley and Sons, Inc., New York, (1972).

APPENDIX A

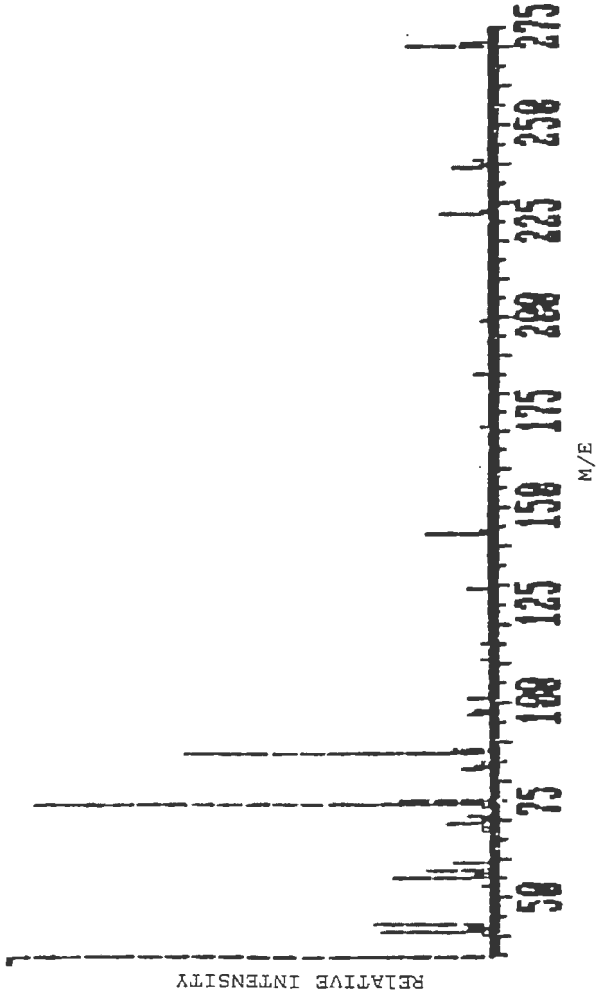
MASS SPECTRA OF STANDARD FATTY ACID METHYL ESTERS



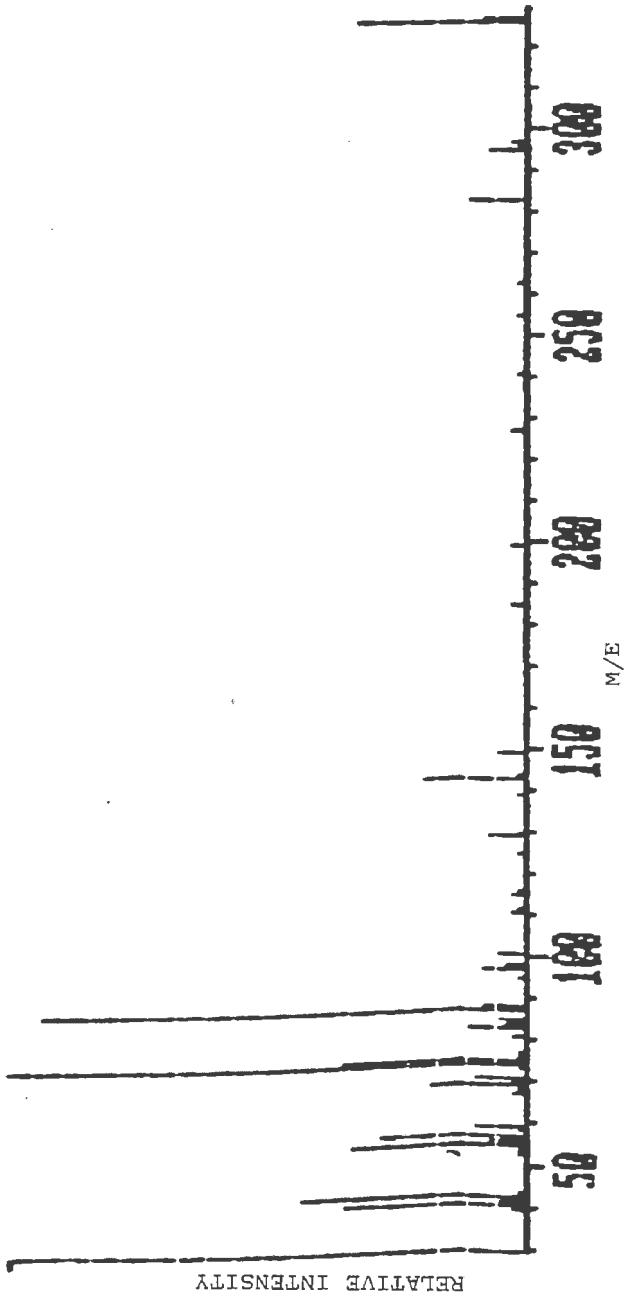
Mass Spectra of Lauric Acid Methyl Ester



Mass Spectra of Myristic Acid Methyl Ester



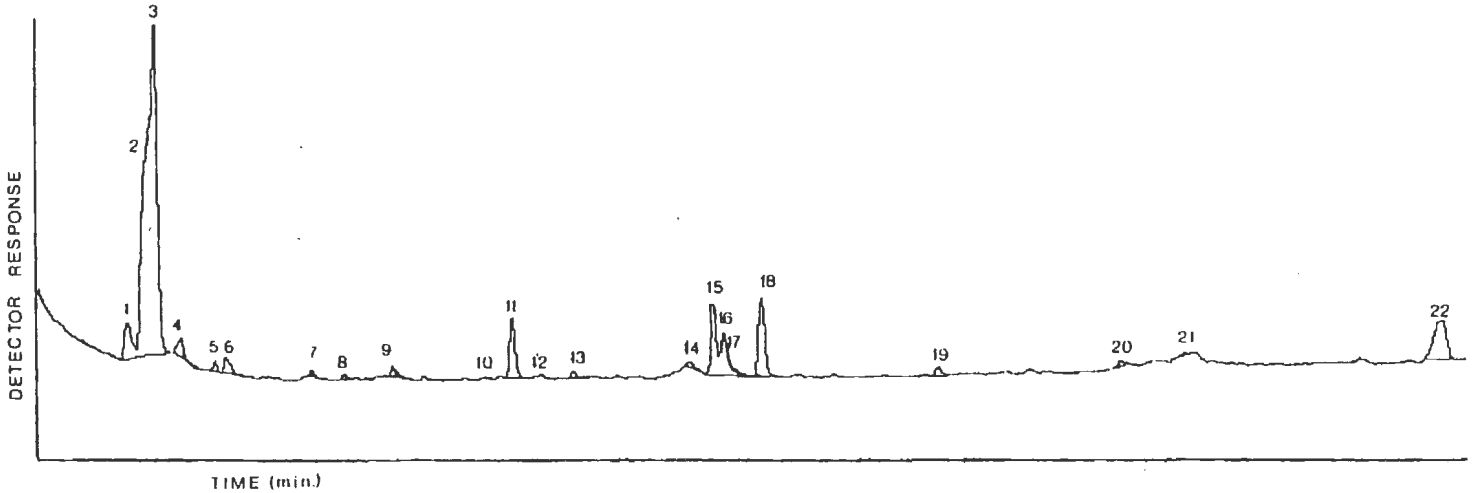
Mass Spectra of Palmitic Acid Methyl Ester



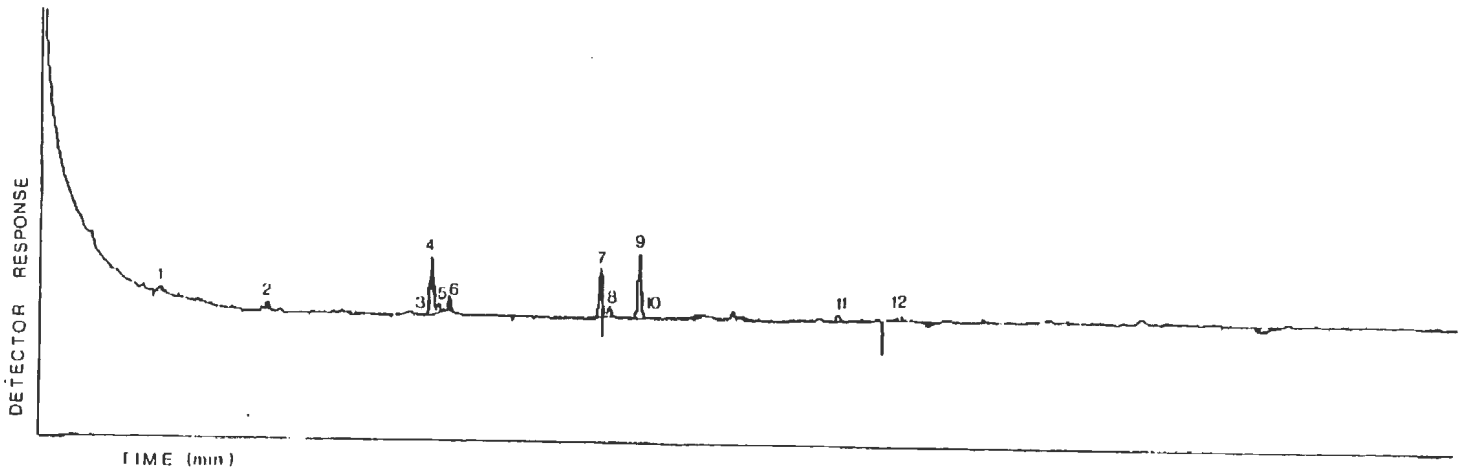
Mass Spectra of Arachidic Acid Methyl Ester

APPENDIX B

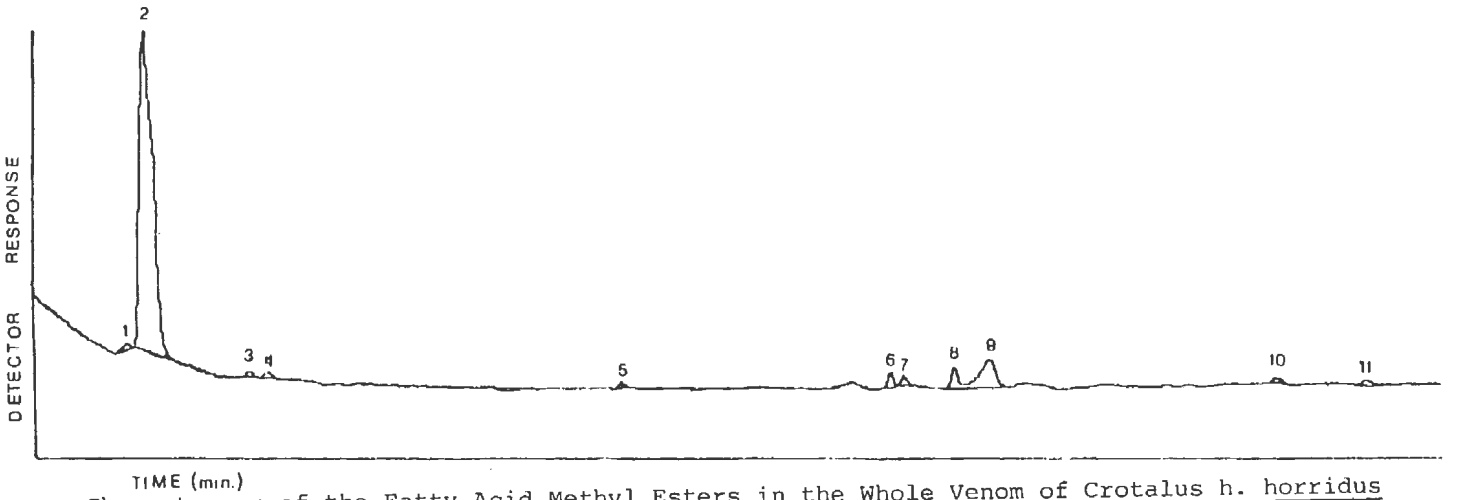
CHROMATOGRAMS COMPARING VENOM LIPID FATTY ACIDS



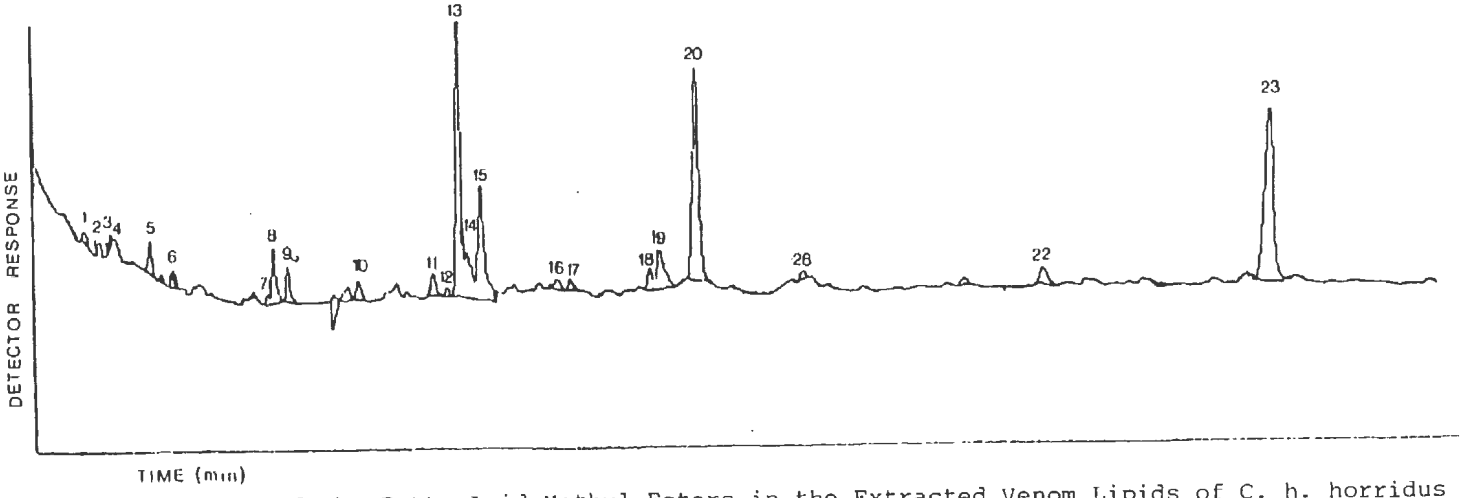
Chromatogram of the Fatty Acid Methyl Esters in the Whole Venom of Crotalus Atrox



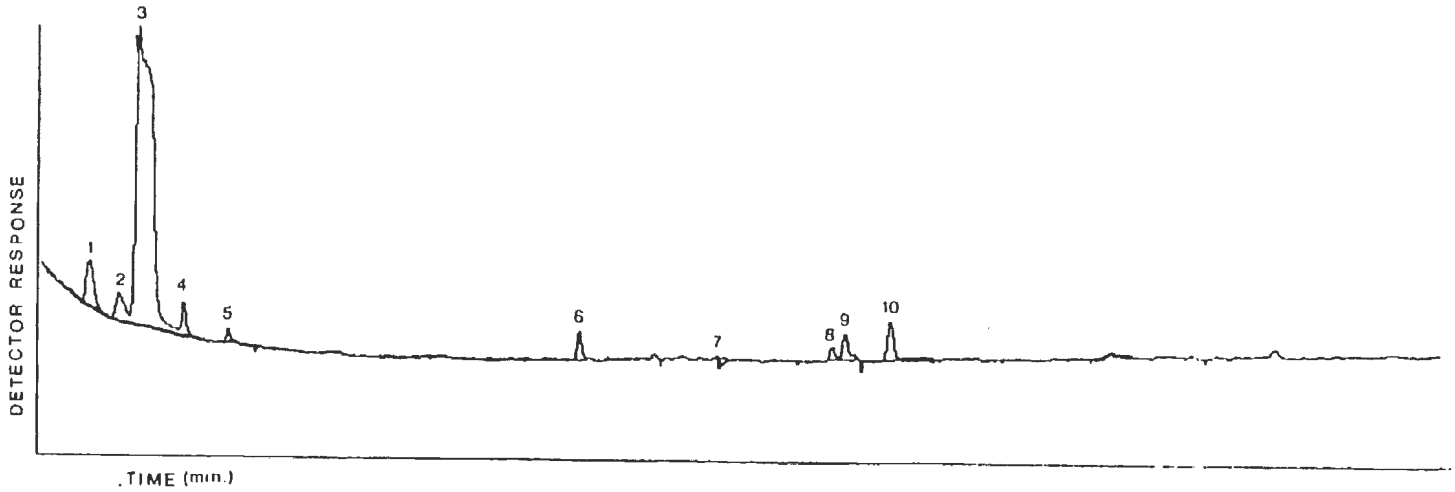
Chromatogram of the Fatty Acid Methyl Esters in the Extracted Venom Lipids of C. atrox



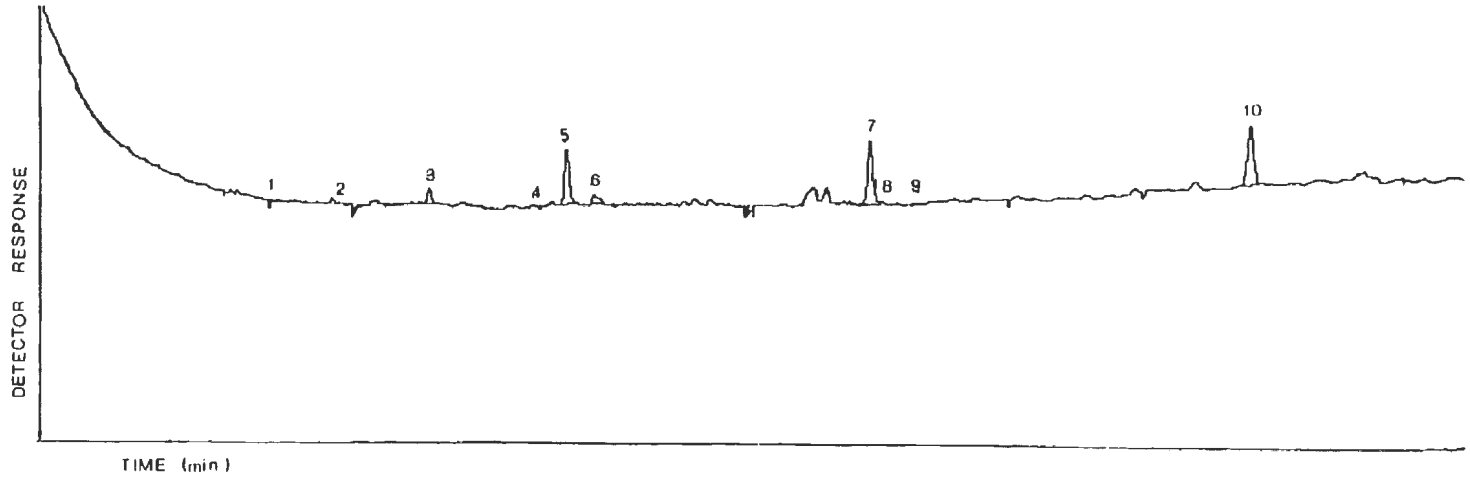
Chromatogram of the Fatty Acid Methyl Esters in the Whole Venom of Crotalus h. horridus



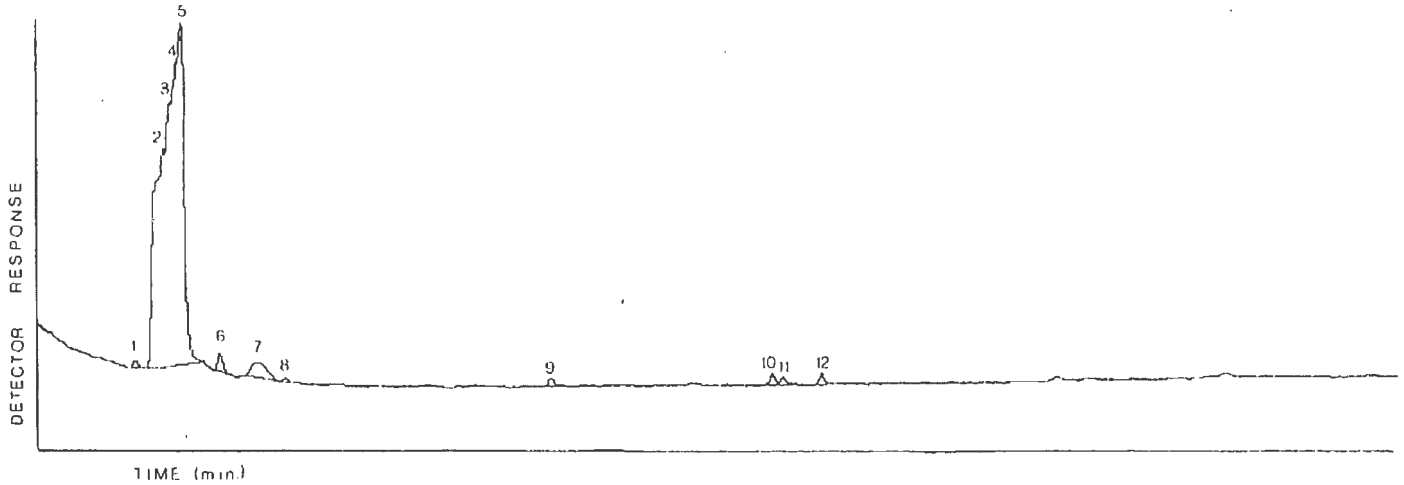
Chromatogram of the Fatty Acid Methyl Esters in the Extracted Venom Lipids of C. h. horridus



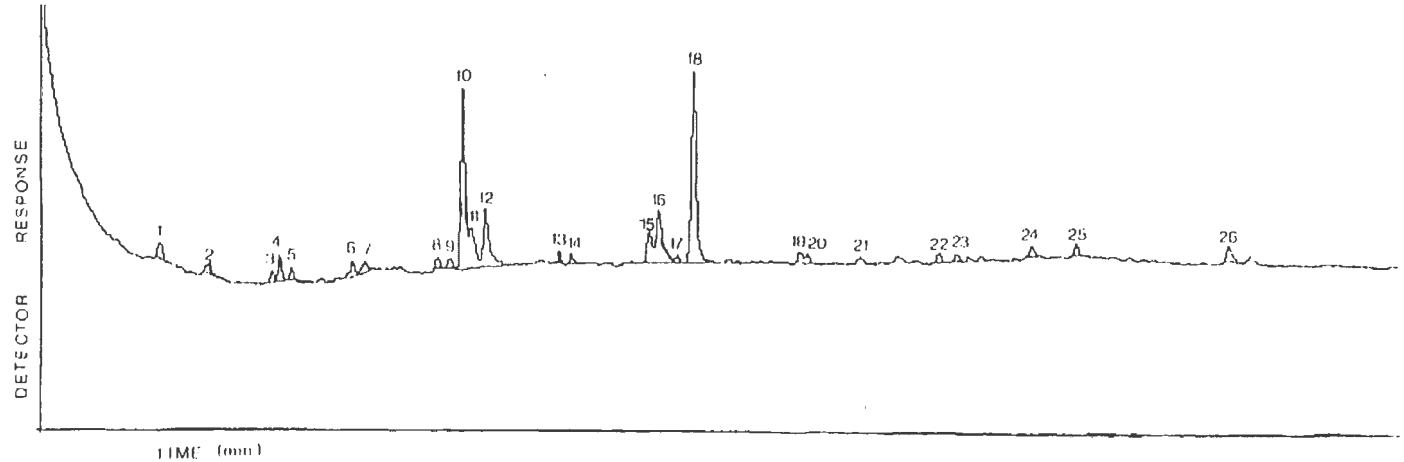
Chromatogram of the Fatty Acid Methyl Esters in the Whole Venom of Agkistrodon c. contortrix



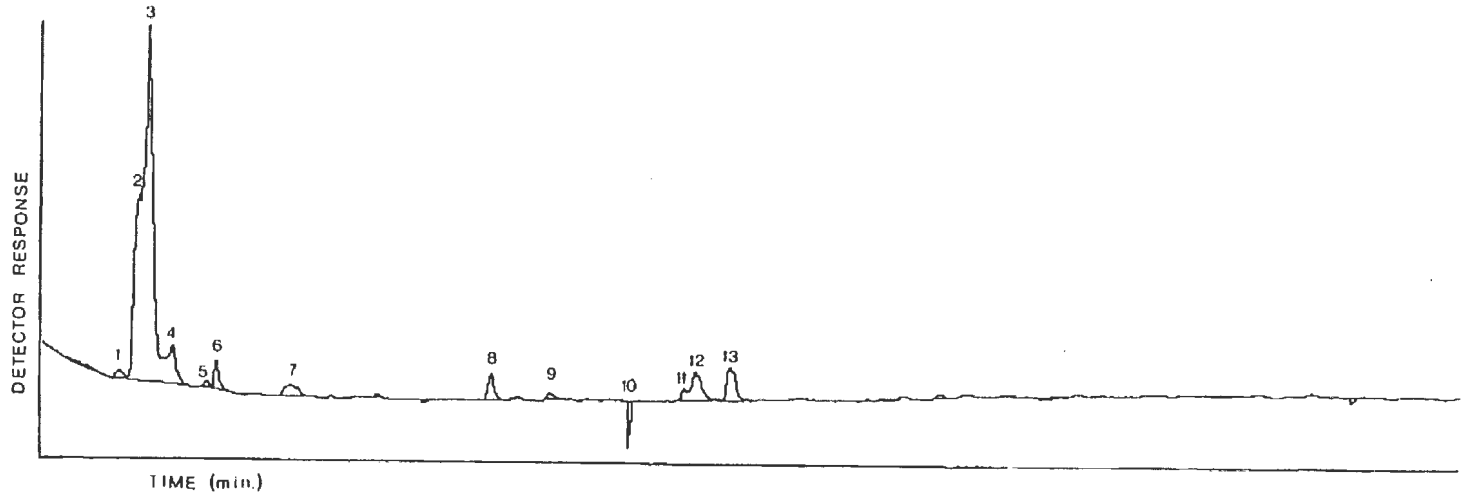
Chromatogram of the Fatty Acid Methyl Esters in the Extracted Venom Lipids of A. c.contortrix



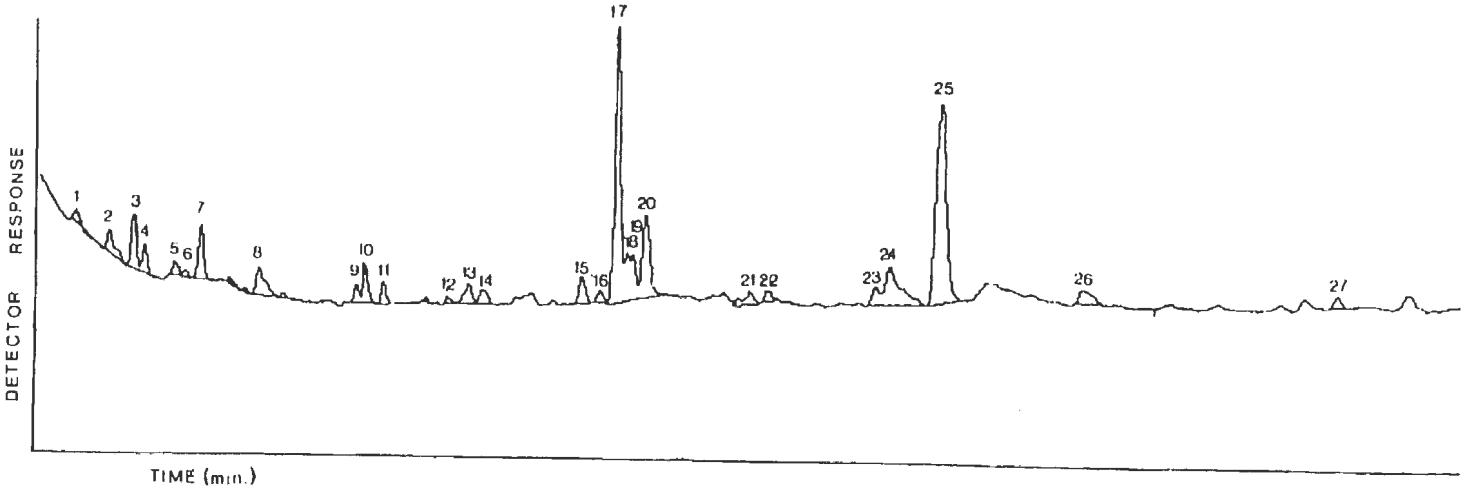
Chromatogram of the Fatty Acid Methyl Esters in the Whole Venom of Crotalus Adamanteus



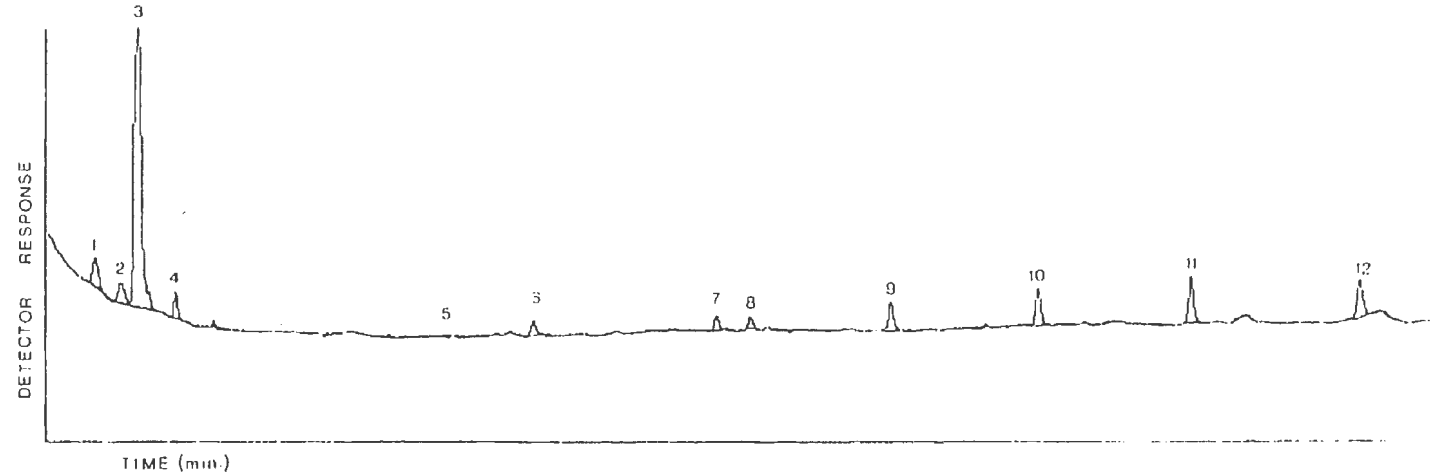
Chromatogram of the Fatty Acid Methyl Esters in the Extracted Venom Lipids of C. Adamanteus



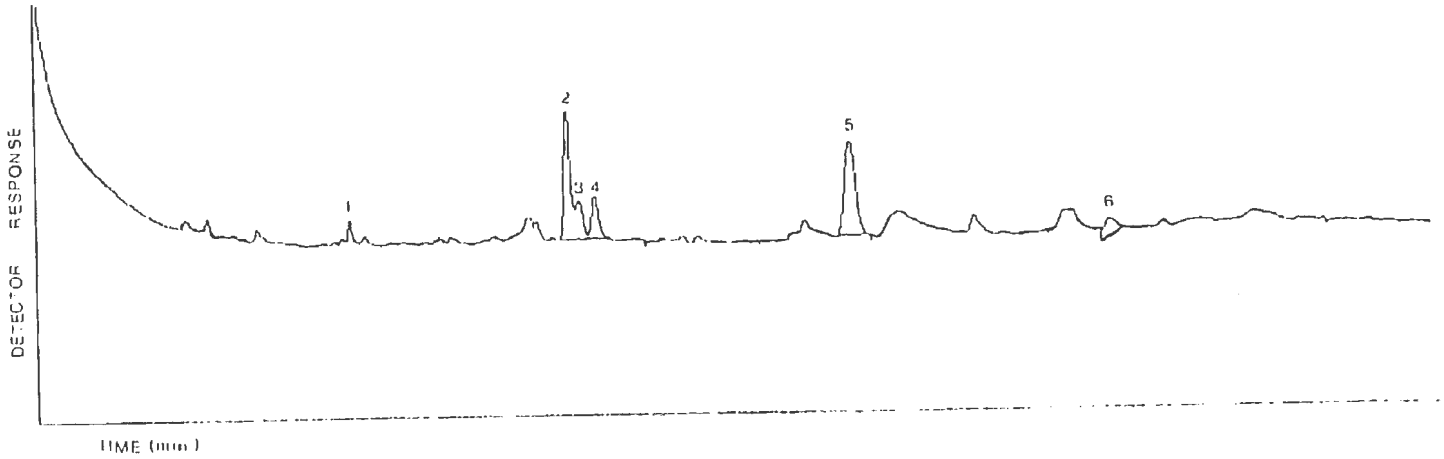
Chromatogram of the Fatty Acid Methyl Esters in the Whole Venom of Agkistrodon p. piscivorus



Chromatogram of the Fatty Acid Methyl Esters in the Extracted Venom Lipids of A. p. piscivorus



Chromatogram of the Fatty Acid Methyl Esters in the Whole Venom of Bothrops asper



Chromatogram of the Fatty Acid Methyl Esters in the Extracted Venom Lipids of B. asper

VITA

Mary Ellen Cooke

Candidate for the Degree of

Master of Science

Thesis: CHARACTERIZATION OF THE FATTY ACIDS IN THE LIPIDS OF SNAKE VENOM

Major Field: Biochemistry

Biographical:

Personal Data: Born February 20, 1960 in Hobbs, New Mexico, the daughter of Keith L. Cooke and Virginia E. Cooke.

Education: GED December 1978, taken at Southeastern Oklahoma State University; recieved Bachelor of Science Degree in Chemistry from Southeastern Oklahoma State University in May, 1983; completed requirements for the Master of Science degree at Oklahoma State University in December, 1986.

Professional Experience: Undergraduate Research Assistant, Department of Chemistry, May, 1980, to May, 1983; Undergraduate Research Assistant, Department of Chemical Engineering, University of Oklahoma, May, 1981, to August, 1981; Graduate Research Assistant, Department of Biochemistry, August, 1984, to August, 1986; Teaching Assistant, Department of Biochemistry, January, 1986, to May, 1986.

Organizations: American Chemical Society, 1981 to 1983; Phi Alpha Theta, 1982 to 1983; Alpha Chi, 1983; Who's Who in American Colleges and Universities, 1983; Phi Lamda Upsilon, 1984.