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MASS SPECTRAL INVESTIGATION OF BIOGENIC AMINES IN THE JELLY FISH POLYORCHIS PENICILLATUS

by

KYUNG-HYUN GAHM

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MASS SPECTRAL INVESTIGATION OF BIOGENIC AMINES IN THE JELLY FISH POLYORCHIS PENICILLATUS submitted by KYUNG-HYUN GAHM in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

P. KEBARLE, Supervisor

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F. PASUTTO

DATE May 15, 88

ABSTRACT

Several biogenic amines, metabolites, and related compounds were derivatized with trifluoroacetic anhydride(TFAA) and were introduced into the GC-MS. The mass spectra and the sensitivity of these compounds were investigated in positive chemical ionization(PCI) where methane or ammonia was used as a reagent gas, electron impact ionization(EI), and negative ion chemcal ionization(NICI), where methane was used as a reagent gas. Every spectrum was characteristic to a compound used, and it provides the information about molecular weight and molecular structure. The sensitivity order was found to be NICI > EI > PCI (methane) > PCI (ammonia). The ammonia PCI was found the least sensitive among all ionization modes used, but all biogenic amines give $[M+NH_4]^+$ as a base peak. Therefore, this ionization mode clearly provides the information about the molecular structure and molecular weight of an analyte. The methane PCI, which is more sensitive than ammonia PCI, gives $[M+H]^+$ or $[MH-TFAOH]^+$ as a base peak to support the molecular structure of an analyte. Although the sensitivity of EI is lower than negative ion chemical ionization, it gives numerous fragment peaks containing abundant information about the molecular structure. The methane negative ion chemical ionization, which was found to be the most sensitive among all ionization modes used in this present study, made it possible to identify picomole quantities of biogenic amines in biological

samples.

By combination fused silica capillary column gas chromatography mass spectrometry and HPLC-EC, which was also found highly specific and sensitive to catecholamines, picomole levels of dopamine were found to exist in mg. wet weight of a nerve-rich tissue of Polyorchis penicillatus. The finding of the existence of dopamine in the nerve rich tissue of Polyorchis penicillatus strongly suggests that this animal has a neurotransmitter in its nervous system.

ACKNOWLEDGEMENTS

I wish to appreciate most sincerely to my supervisor Professor P. Kebarle for his helpful advice and patient supervision throughout the duration of this work.

I wish to acknowledge the assistance and valuable discussion with my coworker, Jun-Mo Chung, who is currently studying Neurophysiology in Department of Zoology, University of Alberta. The procedures including homogenization, alumina extraction and HPLC-EC were developed and performed by him. Figure 4.1 and Figure 4.2, and Table 4.1 to Table 4.3 were kindly provided by him. He is responsible for the work and interpretation of the results shown in these figures.

I wish to thank the members of the Mass Spectrometer group for obtaining the mass spectra.

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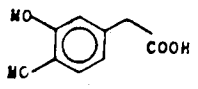
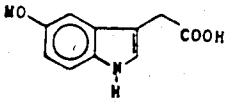
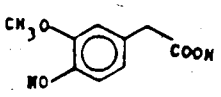
LIST OF ABBREVIATIONS

CA	Catecholamine
CI	Chemical Ionization
DA	Dopamine
DHBA	Dihydroxybenzylamine
DHPG	Dihydroxyphenylglycol
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic Acid 
EC	Electrochemical Detection(Detector)
EDTA	Ethylenediaminetetraacetic Acid
EI	Electro Impact
EN	Epinephrine
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
5-HIAA	5-Hydroxyindoleacetic Acid 
HPLC	High Performance Liquid Chromatography
5-HT	5-Hydroxytryptamine (Serotonin)
HVA	Homovanillic Acid 
LC	Liquid Chromatography
MS	Mass Spectrometry
NE	Norepinephrine
NICI	Negative Ion Chemical Ionization
NMDA	N-Methyldopamine

M-OCT	Meta-Octopamine
P-OCT	Para-Octopamine
PCI	Positive Chemical Ionization
PFP	Pentafluoropropionic group(CF_3CF_2CO)
PFPA	Pentafluoropropionic Anhydride($CF_3CF_2COOCOCF_2CF_3$)
PFPO	CF_3CF_2COO
PFPOH	CF_3CF_2COOH
SALS	Salsolinol
SIC	Selected Ion Current
SIM	Selected Ion Monitoring
SYNE	Synephrine
TFA	Trifluoroacetyl group(CF_3CO)
TFAA	Trifluoroacetic Anhydride($CF_3COOCOCF_3$)
TFAO	CF_3COO
TFAOH	CF_3COOH
$TFANH_2$	CF_3CONH_2
TIC	Total Ion Current
TLC	Thin Layer Chromatography
TRA	Tryptamine
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet Photometric Detection

CHAPTER 1

INTRODUCTION

The naturally found catecholamines, norepinephrine (NE, noradrenaline (I)), epinephrine (EN, adrenaline (II)), dopamine (DA (III)), and tryptamine (TRA (IV)) and related compound, 5-hydroxy-tryptamine (5-HT, serotonin (V)), see Table 1.1, play a role as neurotransmitters in the central and peripheral nervous systems [1,2]. They also play a vital role in human disease. Thus, dysfunctions of catecholamines, and the related compounds, meta(or para)-octopamine (m-OCT (VI) and p-OCT (VII)), synephrine (SYNE (VIII)), 3,4-dihydroxy-phenylalanine (DOPA (IX)), are known to lead to Parkinson's disease[3], depression[4], schizophrenia[5], and pheochromocytoma [6].

The physiology and pharmacology of biogenic amines and their roles as hormones or neurotransmitters have been studied extensively and the discovery of their catabolic pathways has revolutionized this area of biochemical research and led to a growing interest in the analysis of the parent amines, their precursors and metabolites. The major metabolic pathway for the catecholamines may be represented by a single scheme (Fig. 1.1)[7].

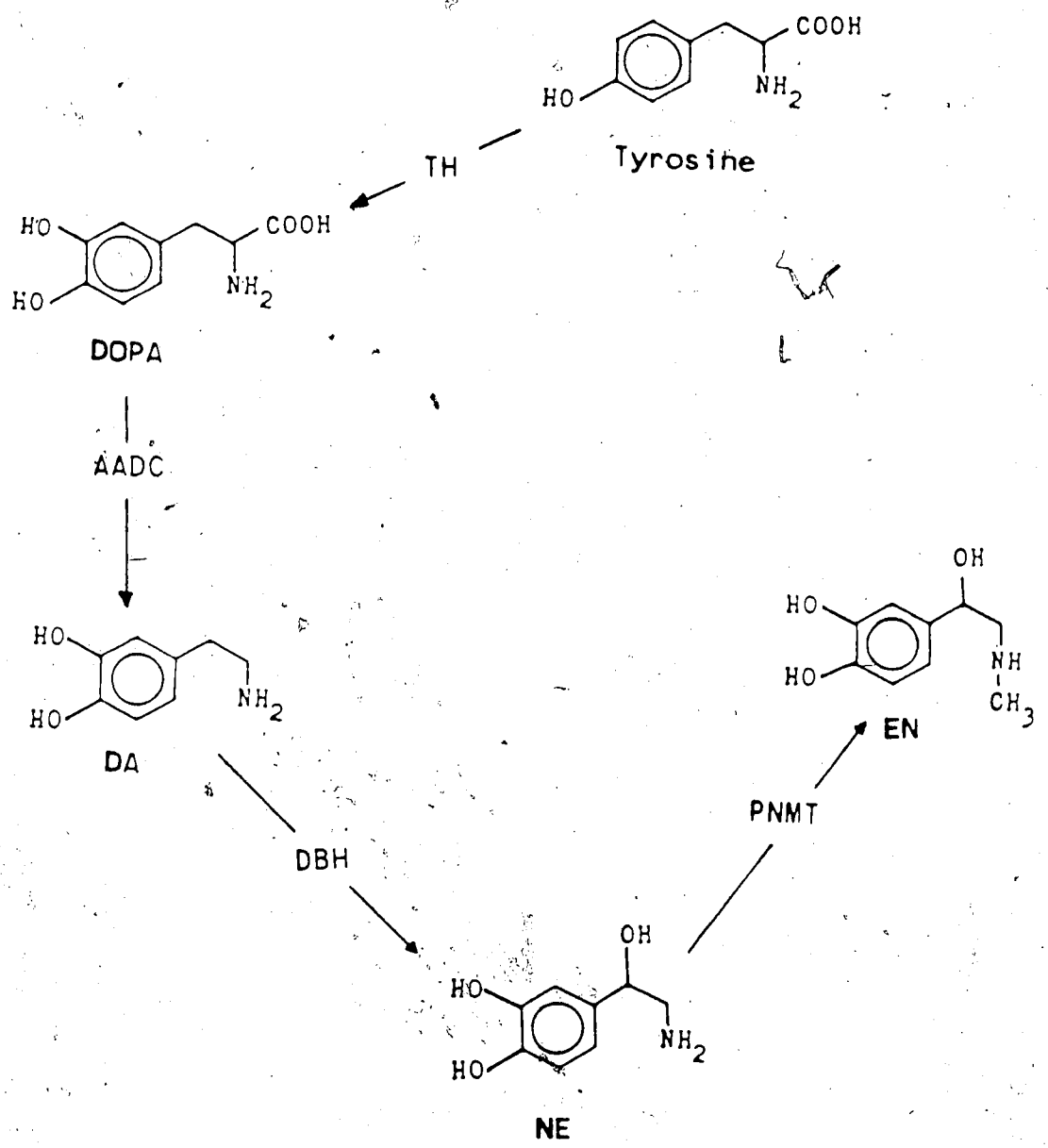
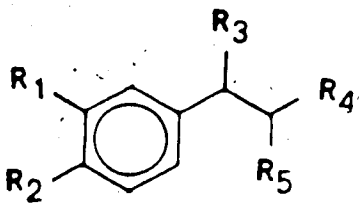
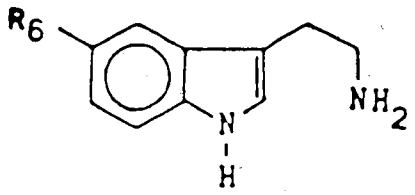


Figure 1.1 Catecholamine metabolism and the related enzymes. TH:tyrosine hydroxylase, AADC:aromatic L-amino-acid decarboxylase, DBH:dopamine b-hydroxylase, PNMT:phenylethanolamine N-methyltransferase.

Table 1.1 Structures of several biogenic amines and metabolites





	R_1	R_2	R_3	R_4	R_5	R_6
(I)	-OH	-OH	-OH	-H	$-NH_2$	-
(II)	-OH	-OH	-OH	-H	$-NH-CH_3$	-
(III)	-OH	-OH	-H	-H	$-NH_2$	-
(IV)	-	-	-	-	-	-H
(V)	-	-	-	-	-	-OH
(VI)	-OH	-H	-OH	-H	$-NH_2$	-
(VII)	-H	-OH	-OH	-H	$-NH_2$	-
(VIII)	-H	-OH	-OH	-H	$-NH-CH_3$	-
(IX)	-OH	-OH	-H	-COOH	$-NH_2$	-

Since progress in research often depended upon development of improved analytical procedures, introduction of improved analytical technology makes new developments in biogenic amine research possible. A variety of analytical methods have recently been developed for the determination of biogenic amines and their metabolites. Among these methods, high performance liquid chromatography with electrochemical detection [8-11] and capillary gas chromatography chemical ionization mass spectrometry [12,13] afford a combination of great selectivity and sensitivity which is not easily obtained by other methods.

The recent availability of combined liquid chromatography mass spectrometry[14-16], which can be used in the selected ion monitoring mode, clearly offers new opportunities to extend further the use of mass spectrometry in biogenic amine research.

In the present study, the mass spectra of several biogenic amines and related compound in various ionization modes in GC-MS were investigated first. Then this technique was applied to the identification of biogenic amines in the jelly fish Polyorchis penicillatus.

The phylogenetic tree is shown in Fig. 1.2. The coelenterate phylum is the most primitive, which possesses a nervous system. In this phylum there are three different classes, Hydrozoa, Scyphozoa, and Anthozoa (Fig. 1.3). The species, Polyorchis penicillatus belongs to the Hydrozoa. To find out whether neurotransmitters are present and how these compounds act in this primitive animal will help us better understand its nervous system.

1.1. GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

Specificity and sensitivity, the two main performance characteristics of combined gas chromatography mass spectrometry(GC-MS), have been utilized in a remarkable number of applications in all areas of biogenic amines research [17-20]. GC-MS represents one of the most powerful and versatile analytical methods available for the unequivocal identification and quantitation of small amounts of organic compounds in complex mixtures. The recently available capillary columns provide highly inert systems to assay labile substances

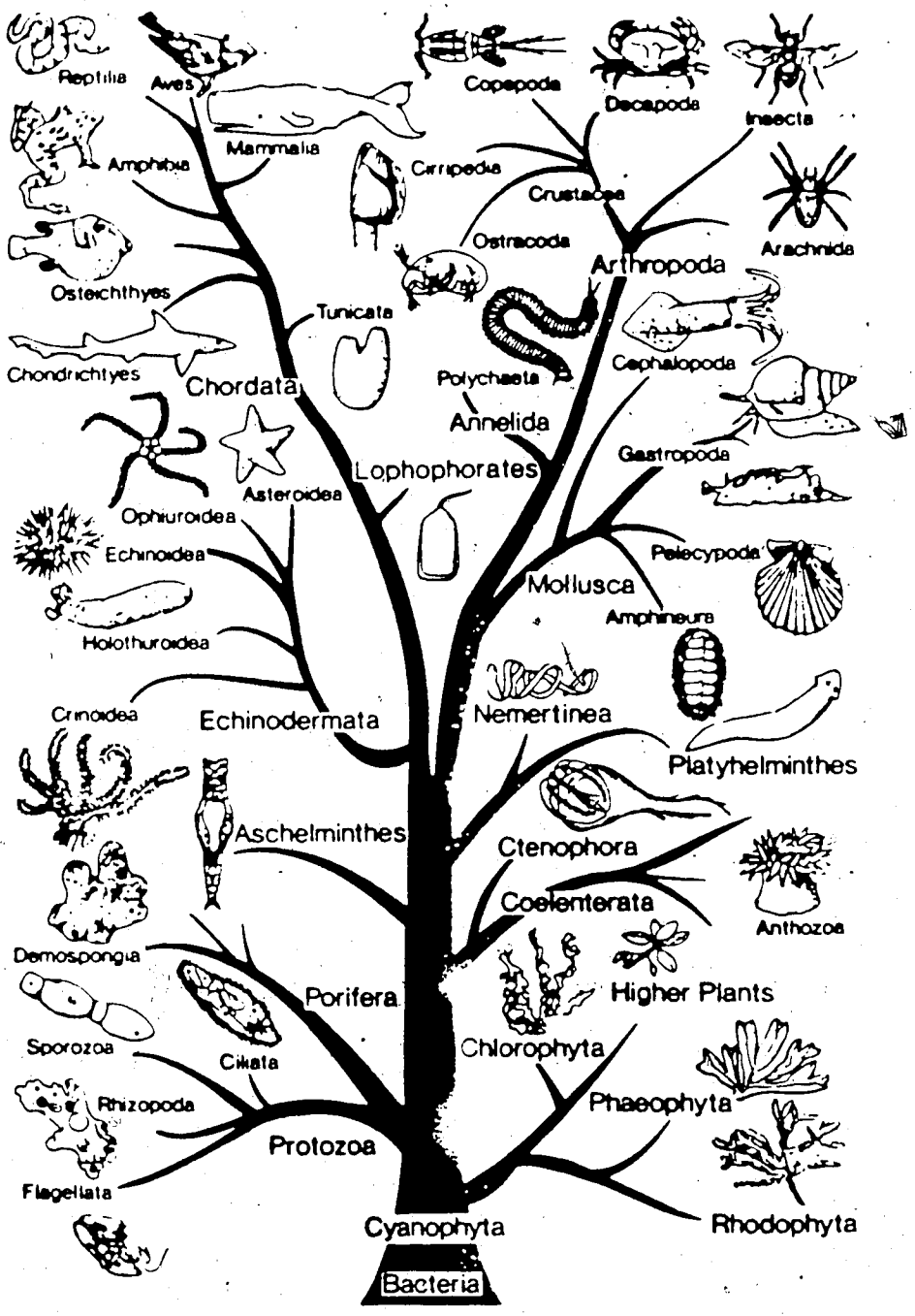


Figure 1.2 The phylogentic tree.

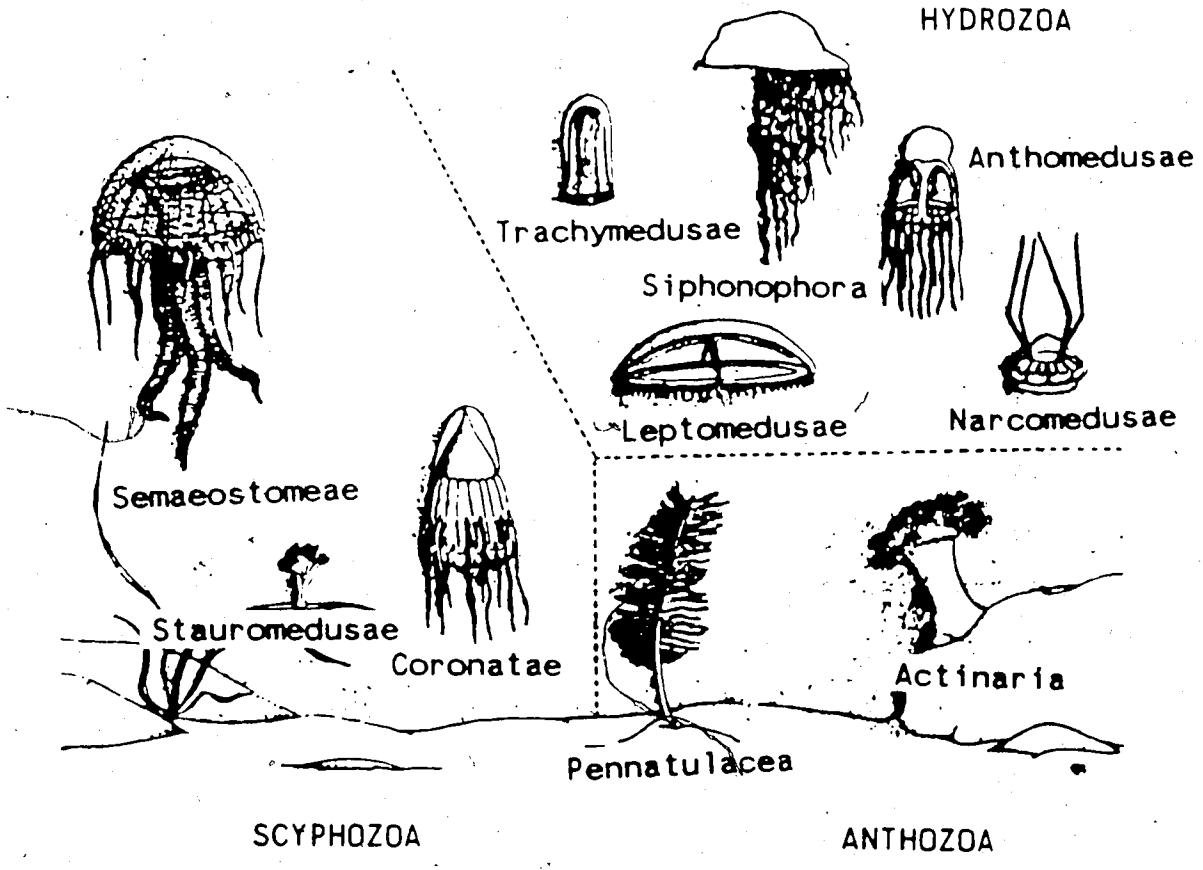


Figure 1.3. Some examples of cnidarian species belonging to the classes Hydrozoa, Scyphozoa, and Anthozoa.

as well as sufficient efficiency and resolution to separate closely related substances in complex samples. The technique of selected ion monitoring(SIM), where the mass spectrometer focuses on a few preselected masses characteristic of the compound rather than scanning the entire mass range, is now used extensively because it permits a considerable relaxation in sample purification requirement and provides confirmation of analyte identity in the case of coelution of one or more compounds with the compounds of interest [21-23].

The sensitivity and specificity of GC-MS can be increased further by the use of the new ionization methods known as positive and negative ion chemical ionization methods. The chemical ionization technique avoids the extensive fragmentation of the analyte molecular ions and thus improves the detectability of molecular ion relative to that obtained with electron impact ionization. Positive chemical ionization(PCI) has been throughly investigated and is being used extensively [24-27]. Negative ion chemical ionization(NICI), a relatively new technique, offers a dramatic increase in sensitivity and permits the quantification of even a few femtograms of amines by GC-MS-SIM [28-31].

1.2. CHEMICAL IONIZATION MASS SPECTROMETRY

In chemical ionization mass spectrometry (CIMS), ions from

the molecule of interest are formed by reactions between the molecule of interest and a set of ions which serve as ionizing reagents.

The essential reactions in positive ion chemical ionization can be given in general form in Reactions (1) to (3). The reactant ions are generated by ionizing the reagent, which exists in large amounts, by electron impact ionization and ionization is followed by ion-molecule reactions. Because the reagent gas is present in large excess, ions from the reagent gas are essentially the only ones produced by electron impact ionization. The reactant ion and the reagent gas neutrals produce the chemical ionization reactant ion or reactant ion series shown below, where e^* means a thermalized electron.

$$e + R \longrightarrow R^+ + e + e^* \qquad (1)$$

$$R^+ + A \longrightarrow A_1^+ + N_1 \qquad (2)$$

$$A_1^+ \longrightarrow A_2^+ + N_2 \qquad (3)$$

$$\longrightarrow A_3^+ + N_3 \qquad (4)$$

$$\longrightarrow A_i^+ + N_i \qquad (i)$$

The reactant ion or reactant ion array represents the stable end products of the ion molecule reactions. Collision of the reactant ions, R^+, with the additive produces an ion, A_1^+, characteristic of the additive (Reaction (2)). The additive is the substance under investigation and is present at low concentration levels. A_1^+ may fragment by one or

more pathways, as in Reaction (3). An advantage of chemical ionization mass spectrometry is the fact that a large variety of reagent gases can be used to change the spectrum of the substance under investigation[32-34].

Formation of negative ions by interaction of electrons and sample molecules can occur by three different mechanisms; resonance electron capture, dissociative resonance capture, and ion-pair production[27,35].

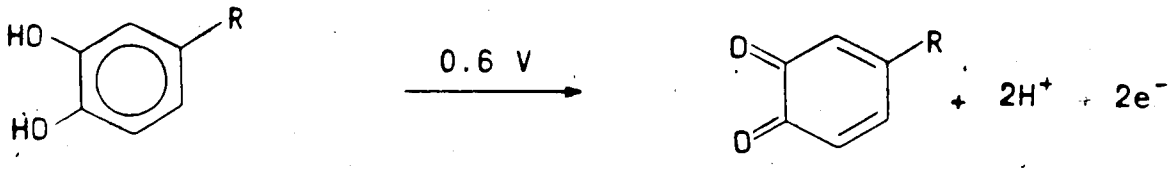
$AB + e^* \longrightarrow AB^-$		Resonance Capture
$AB + e^* \longrightarrow A + B^-$		Dissociative Resonance Capture
$AB + e \longrightarrow A^+ + B^- + e$		Ion-Pair Production

In negative ion chemical ionization mode, thermal or near thermal electrons (e^*) are produced as the reactant (Reaction 1). NICI mass spectrometry can lead to sample ion currents under electron capture conditions that exceed ion currents in the positive by two or three orders of magnitude [27-29]. Enhanced sensitivity in the electron capture negative ion mode will only be realized for sample molecules which possess a positive electron affinity and a large cross section for electron capture. Generally, molecules with positive electron affinity have very large electron capture cross sections. The electron capture negative ion technique has found widespread use in quantitation of trace level mixture components

by combined gas chromatography-mass spectrometry. In this methodology, most organic samples have to be derivatized in order to increase their volatility to facilitate passage of the sample through the GC and to endow the compounds with electron affinity.

1.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION (HPLC-EC)

High performance liquid chromatography with electrochemical detection(HPLC-EC) has become one of the most important methods for the determination of biogenic amines and related compounds [36-38]. Of the HPLC methods, those with ultraviolet photometric detection(UV) are insufficiently sensitive and the fluorimetric detection methods require formation of derivatives, since the fluorescence of the parent substances is not sufficiently intense[39-43]. The electrochemical detector(EC) is extremely sensitive and selective since it only detects compounds that can be electrolyzed by the electrode. Because compounds containing catecholic, phenolic, and indole groups have low oxidation potentials, these compounds in an aqueous solution can be easily oxidized by the application of a suitable potential between two electrodes used in the detector. The typical oxidation reaction occurring in the detection cell is shown in the following scheme:



The abstraction of two electrons from the compound produces a current which then is amplified for detection by the amperometric measurements. Because of the selectivity of electrochemical detection, many background compounds present in the samples do not interfere and thus the sample pretreatment is often simpler than that required by the other methods[37,44,45].

The advantages of HPLC-EC are high resolution and sensitivity combined with good precision, even at low solute concentrations and a selectivity of detection that can be obtained by variation of electrochemical conditions[46].

1.4. THE NERVOUS SYSTEM AND SYNAPTIC TRANSMISSION

The nerve cells, neurons, the essential conducting units of the nervous system, are large cells generally consisting of a cell body containing a nucleus, a number of branching dendrites which extend like antennae from the cell body and provide an enlarged surface area for the reception of signals from other cells, and a single straight axon that conducts electrical

signals away from the cell body to distant targets (Fig. 1.4) [47].

Neurons receive, conduct, and transmit a signal or impulse from one part of the body to another. Even though the signals and impulses may vary a lot, their forms are the same, consisting of changes of the electrical potential across the membrane [48]. Neuronal impulses are transmitted from one cell to another at specialized sites of contact known as synapses (Fig. 1.5) [49,50]. In synapses, there is always a distinct gap between the presynaptic and postsynaptic membranes which is termed the synaptic cleft. The presynaptic cell is electrically separated from the postsynaptic cell by the synaptic cleft. Transmission of a signal through the cleft occurs by either electrical or chemical mechanisms. In electrical transmission, one neuron passes its impulse to another by direct electrical coupling through gap junctions. In chemical transmission, a change of electrical potential in the presynaptic cell triggers the release of a chemical known as a neurotransmitter, which diffuses across the synaptic cleft and provokes an electrical change in the postsynaptic cell. Thus the communication involves converting an electrical signal into a chemical signal and then converting the chemical signal back to an electrical one again[51].

To consider a substance as a neurotransmitter, several different lines of evidence should be provided to meet the

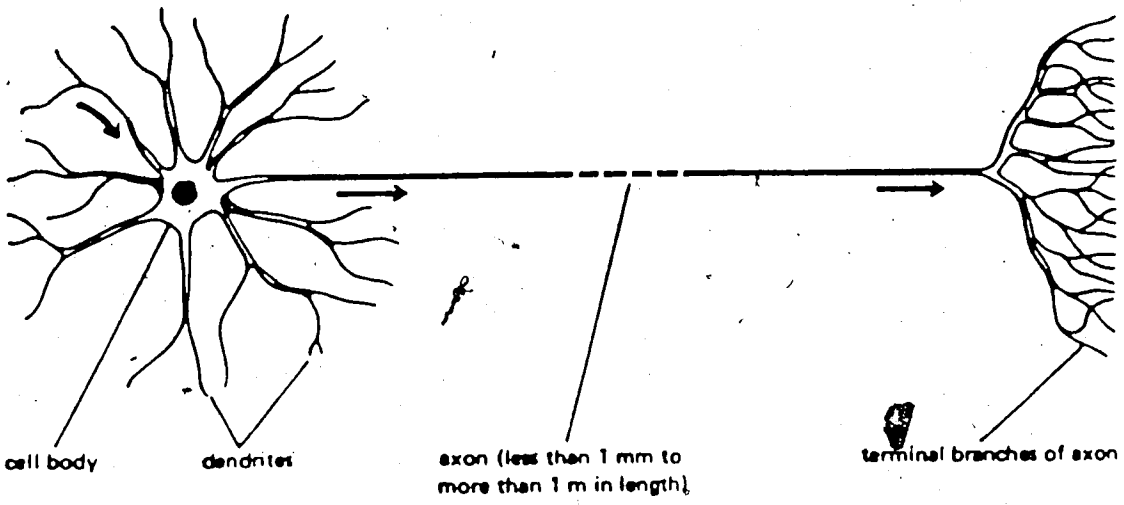


Figure 1.4 Schematic diagram of a typical neuron of a vetebrate. (The arrows indicate the direction in which signals are converged.)

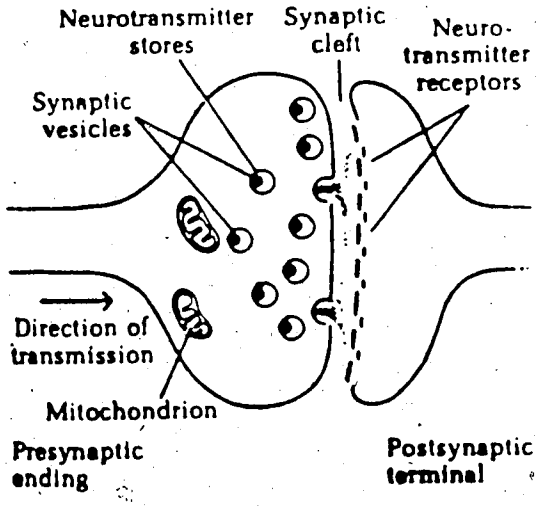


Figure 1.5 Schematic diagram of a typical synapse.

following generally accepted criteria[52-54]. The presynaptic neuron must synthesize, contain, and release the transmitter at a concentration sufficient to produce demonstrable action in a postsynaptic cell. Applying a test drug to the postsynaptic cell should produce the same action as the endogenous neurotransmitter. Finally, there must be a mechanism to remove the transmitter in order to terminate the action of the neurotransmitter[54].

1.5 PREVIOUS EXPERIMENTS TO INVESTIGATE NEUROTRANSMITTERS IN CNIDARIANS

Several attempts have been made to investigate possible neurotransmitters in cnidarian animals. Ostland reported that epinephrine, norepinephrine, and dopamine were not detected in several cnidarians by paper chromatography. However, he reported that there was an unknown catecholamine in the sea anemone Metridium dianthus, which he called "Catechol 4" [55]. It was reported that DOPA, norepinephrine, and dopamine were detected in the sea anemone Actina equina by TLC or ion exchange chromatography or partition paper chromatography after alumina extraction [56]. The existence of dopamine, DOPA and 5-HT in the sea anemone Metridium senile by TLC after organic extraction was also reported . The colors of sample spots did not match with those of the authentic standards [57]. However, Carlberg could not identify any catecholamines and 5-HT except DOPA in

the sea anemone Metridium senile by the HPLC-EC [58]. Venturini et al demonstrated that 5-HT, dopamine and norepinephrine were detected in the hydra Chlorohydra viridissima by HPLC-EC [59].

The difficulty in obtaining conclusive evidence to support the existence of neutransmitters from the previous experiments lies in the fact that the extract of the cnidarian usually contained substances which coeluted with catecholamines and then interfered the normal travel of catecholamines on the chromatographic media. Furthermore since the amines are present in extremely low concentrations in cnidarians, more sensitive and selective detection methods for the identification of the transmitters are required. The more selective and sensitive gas chromatography mass spectrometry(GC-MS) technique can provide much more reliable results in the research on transmitters in cnidarians.

CHAPTER 2

EXPERIMENTAL

2.1. REAGENTS AND CHEMICALS

Trifluoroacetic anhydride (TFAA) was obtained from General Intermediates of Canada. Dopamine(DA) hydrochloride, norepinephrine(NE) hydrochloride, epinephrine(EN) hydrochloride, meta and para octopamine hydrochloride, synephrine(SYNE) hydrochloride, N-methyldopamine(NMDA) hydrochloride, tryptamine(TRA) hydrochloride, 5-hydroxytryptamine(5-HT) hydrochloride, 3,4-dihydroxyphenylalanine(DOPA), dihydroxyphenylgycol(DHPG), 5-hydroxyindoleacetic acid(5-HIAA), homovanillic acid(HVA), salsolinol(SALS), dihydroxyphenylacetic acid(DOPAC), dihydroxybenzylamine(DHBA) and tris(hydroxymethyl)aminomethane(TRIS) were from Sigma Chemical Co. (St. Louis, MO). Sodium octyl sulfate(SOS) was from KODAK Laboratory Chemicals (Rochester N.Y.) Other chemicals were chromatographic or reagent grade and were obtained from local suppliers.

2.2. GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

The GC-MS system consisted of a gas chromatograph (Varian, Vista 6000) equipped with a SPB-5 fused silica capillary column (30m length; 0.25 um film thickness) and a VG 7070E mass spectrometer equipped with an EI and CI source with negative ion chemical ionization capability. The end of the capillary column was inserted directly into the ion source of the mass spectrometer. Helium was used as the carrier gas with a flow rate of about 1.5ml per minute. The temperature of the GC oven was kept at 100 ^{o}C initially and increased to 240 ^{o}C (or 290 ^{o}C) by 10 ^{o}C per minute. The injector and the interfacial region between gas chromatograph and mass spectrometer were maintained at 240^{o}C.

The mass spectrometer was operated at a resolution R=1000. The ionization energy was 150 eV and emmision current was 5 mA for the chemical ionization mode. 40 eV was used for the electron impact ionization. The ion source temperature was maintained at 190 ^{o}C. A PDP-11/73 computer was used to acquire the data. The mass spectrometer was set to scan mass unit 130 up to 700 when NICI spectra were recorded since there was great interference by the Iodide ion at mass unit 127 in the negative chemical ionization mode. To take NICI spectra, methane was used for the reagent gas. Spectra were also obtained in the electron impact ionization and positive ion chemical ionization modes where methane and ammonia were used

as the reagent gases in order to study the fragmetation patterns and compare the relative sensitivity between these ionization modes.

2.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS

Two liquid chromatography systems (system I and II) were used in the present study. The chromatography system I was set up and operated in Department of Zoology, University of Alberta by my coworker Jun-Mo Chung. The chromatography system II was developed for the coupling experiment of a HPLC to MS-MS. No additives(sodium octyl sulfate and disodium EDTA) were used in the mobile phase of the chromatography system II since the additives in chromatography system I caused great interference in the mass spectra. An electrochemical detector and a UV detector were employed in chromatography system I and II, respectively.

2.3.1. CHROMATOGRAPHY SYSTEM I

A. HPLC INSTRUMENTATION

HPLC determinations were performed with a WATERS MODEL 6000 (Milford, MA, U.S.A.) liquid chromatograph equipped with a Rheodyne 7125 injection valve with a 200 ul sample loop, an

Econosphere RP-18 column (5 um particle size, 250 mm x 4.7 mm) from Econosphere, and BAS LC-4B amperometric detector with a TL-5 glassy carbon electrode maintained at a potential of 0.75V vs. a Ag/AgCl reference electrode with a sensitivity of 2 nA/V.

B. CHROMATOGRAPHIC CONDITIONS

The mobile phase consisted of 55 mM sodium phosphate monobasic, 0.85 mM sodium octyl sulfate (SOS) which was added to act as an ion-pairing agent [60,61], 7 mM disodium EDTA which was added to reduce the detector noise and to protect the solutes from oxidation, and 9% acetonitrile. The pH of the mobile phase was adjusted to 3.75 with phosphoric acid. The mobile phase was filtered through a 0.22 um membrane filter (Millipore, Bedford, MA, U.S.A.) and then degassed under vacuum before use. A flow rate of 0.5 ml/min. at ambient temperature was employed in the present study.

2.3.2. CHROMATOGRAPHY SYSTEM II

A. HPLC INSTRUMENTATION

A Waters model 6000A liguid chromatograph pump, a Rheodyne model 7125 injection valve with a 100 ul sample loop, and a Waters model 440 fixed wavelength UV detector (254 nm) were used for the HPLC separation. The HPLC chromatogram was recorded on

a chart recorder (Yokogawa, Tokyo, Japan). A reversed phase Supelco LC-18-S (4.6 mm x 250 mm) analytical column was used.

B. CHROMATOGRAPHIC CONDITIONS

The mobile phase consisted of 0.02 M ammonium phosphate (mono basic) and 10% methanol. The pH of the mobile phase was adjusted to 3.75 with 1M HCl. The mobile phase was filtered through a 0.22 um membrane filter (Millipore, Bedford, MA, U.S.A.) and then degassed under vacuum before use. A flow rate of 0.5 ml/min. at ambient temperature was employed in the present study.

2.4. ANALYTICAL SYSTEMS TO STUDY BIOGENIC AMINES IN THE JELLYFISH POLYORCHIS PENICILLATUS

A scheme of the analytical procedures for the analysis of biogenic amines in the jellyfish is shown in the flow chart fashion in Fig. 2.1. The procedures including homogenization, alumina extraction and HPLC-EC were developed and performed by my coworker, Jun-Mo Chung who is currently studying Neurophysiology in the Department of Zoology, University of Alberta.

The hydrozoan jellyfish Polyorchis penicillatus were collected from the Bamfield Marine Station, Vancouver Island,

B.C., Canada. The nerve rich tissue dissected from the animals was homogenized and centrifuged. 20 ul of the supernatant solution was introduced into the chromatography system II to find out the number of compounds that existed in the solution at concentration down to nanomole levels.

The catecholamines were separated from the supernatant solution of the homogenate with an alumina extraction procedure[62]. The alumina extract was introduced into the chromatography system I to identify and quantify the catecholamines present in the sample. Several alumina extracts were combined and then derivatized with trifluoracetic anhydride (TFAA). The derivatized products were then introduced into the GC-MS. These analytical procedures are described in detail in the following sections.

2.4.1. HOMOGENIZATION AND ALUMINA EXTRACTION

The nerve rich tissues were dissected from jellyfish that were kept unfed for at least two days. The no feeding step was necessary because the nerve rich tissue consisted of a strip of tissue which included nerve rings in the velum and some ring canal tissue which might contain catecholamines from prey organisms ingested by the jellyfish. The general structure of a jellyfish with one quadrant cut away is shown in Fig 2.2. The combined tissue samples from 1 to 3 animals were weighed and

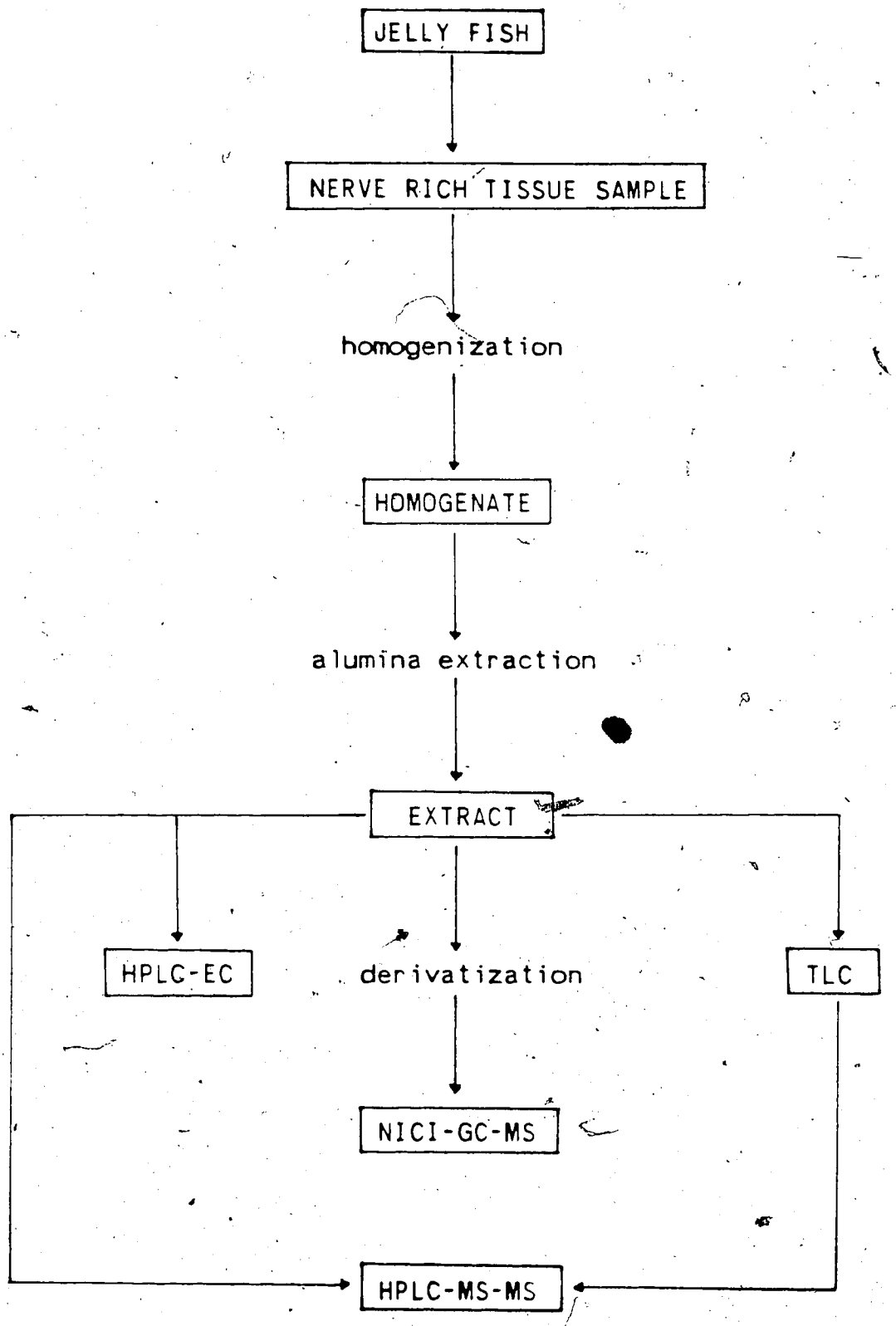


Figure 2.1 Scheme of the analytical procedures for the analysis of biogenic amines in the jellyfish

immediate y frozen at -25 ^{o}C to prevent the action of enzymes if such were present. 1 ml of freshly prepared ice-cold 0.1 N perchloric acid containing 1% sodium metabisulfate and internal standard (DHBA: dihydroxybenzylamine) were added to the frozen sample to denature the proteins and to protect catecholamines from oxidation. The samples were homogenized by hand or ultrasonication, and centrifuged at 3600 g for 1 hour at 4 ^{o}C to remove the cell debris and proteins. The supernatant was transferred to the test tube and 50 mg of activated alumina, which had been baked at 120 ^{o}C for 2 hours and washed with acid according to the procedure described by Anton and Sayer[62], was added to the test tube in order to extract the catecholamines. The separation of the catecholamines depends on the fact that the free bases, which are formed in a basic solution, are adsorbed on the alumina. In an acidic solution desorption from the alumina occurs since the much more stable ammonium salts are formed in solution. 1 ml of TRIS buffer (1.5 M of tris(hydroxymethyl)aminomethane) was added and the test tube was then vortexed for 5 minutes to adsorb catcholamines on the alumina, and centrifuged for 30 seconds at 2500 rpm. The solution was removed by vacuum aspiration and the alumina was washed twice with double distilled water as quickly as possible because the catecholamines are known to be unstable in the alkaline solution. 100 ul of 1.0 M of HCl solution was added to the test tube and then the test tube was vortexed again to elute the catecholamines from the alumina. The test tube was centrifuged for 30 seconds and the acidic extract was pipetted

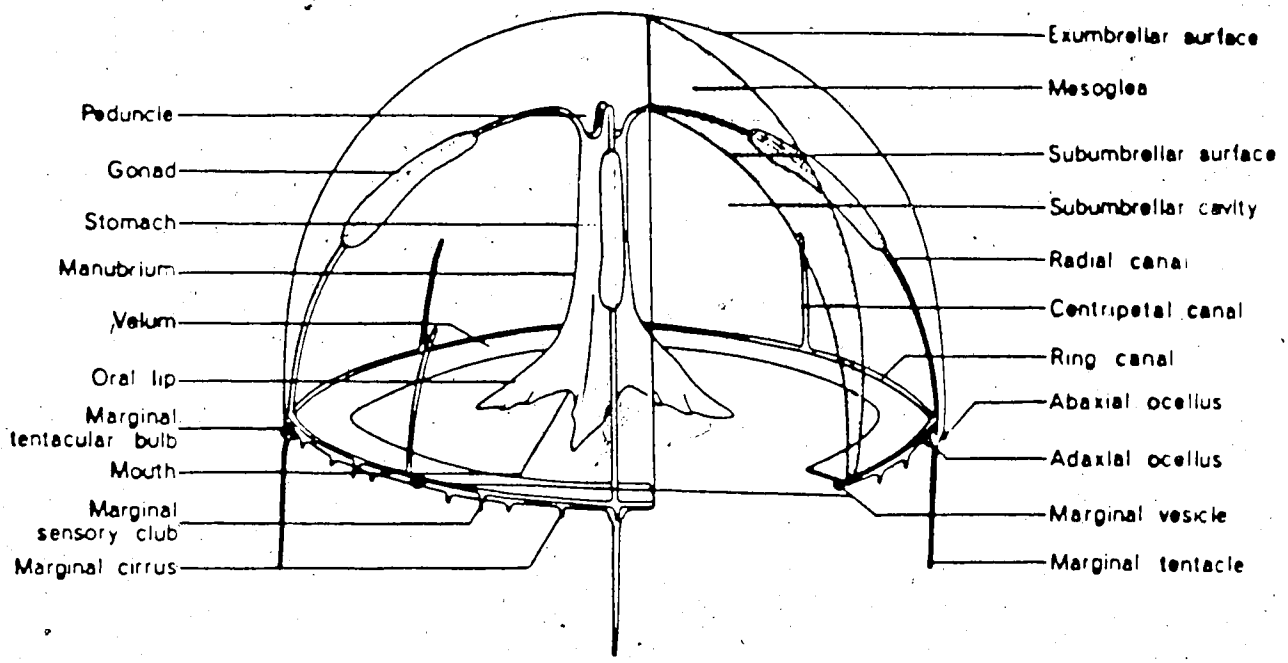


Figure 2.2 General structure of a hydromedusa with one quadrant cut away.

to a 500 ul container and stored in the refrigerator until assayed. 30, 50 or 100 ul of the acidic extract was injected into the HPLC to analyze for biogenic amines in the jellyfish. Several alumina extracts were combined together to obtain sufficient amounts of catecholamines (approximately 80-100 pmole) and then the solvent was evaporated under reduced pressure. The residue was derivatized as described under "Derivatization" and then subjected to GC-MS analysis.

For analytical comparisions working standard solutions of 80 nM of given authentic standards in 0.02 M HCl solution were prepared immediately before use by dilution of stock solutions of the given reference amine and internal standard (0.1 M). These solutions were prepared monthly in 0.02 M HCl solution which contained 0.54 mM EDTA disodium salt, and stored in brown bottles at 0 to -5 ^{o}C.

2.4.2. DERIVATIZATION

The combined alumina extract or a given standard solution was evaporated with a gentle stream of nitrogen under reduced pressure. 100 ul of ethyl acetate and 100 ul of TFAA were added to the dried residue, and the mixture was then allowed to stand to react for one hour at room temperature. The mixture was evaporated again to remove excess reagents with a stream of

nitrogen and 1 ml of ethyl acetate was added to the residue. The ethyl acetate solution was stored in the freezer at - 14 ^{o}C until assayed. 3, 1, or 0.5 ul of this solution was introduced into the NICI-GC-MS.

To obtain individual spectra of the authentic compounds, 10-50 mg of the pure catecholamine was weighed and the same procedure was applied as above.

CHAPTER 3

INVESTIGATION OF MASS SPECTRA OF SEVERAL BIOGENIC AMINES AND DHPG IN VARIOUS IONIZATION MODES OF GC-MS

In order to achieve a certain experience in the analysis of biogenic amines as a preparation towards the possible identification of biogenic amines in jellyfish, this author undertook a more general investigation of the GC-MS capability for the biogenic amine analysis.

Several biogenic amines and DHPG were derivatized with trifluoroacetic anhydride(TFAA) and introduced into the GC-MS. The spectra were recorded: (a) in positive ion chemical ionization mode where methane or ammonia was used as a reagent gas; (b) with electron impact ionization, and (c) with negative ion chemical ionization where methane was used as a reagent gas.

3.1. NEED FOR DERIVATIZATION

A molecule must be sufficiently nonpolar, volatile and thermally stable in order to pass through the gas chromatograph column. However, few biogenic amines and related compounds meet

these requirements. Therefore, the acidic hydrogens in the polar groups should be replaced with an appropriate group to form a single chemically stable and nonpolar derivative. In principle, the techniques developed for making derivatives for use in gas chromatography[63] should be also useful in GC-MS. The use of acylating agents containing fluorine atoms was developed for the sensitive gas chromatographic electron capture assays of several biogenic amines. In particular, the perfluoro acid anhydrides, trifluoroacetic anhydride(TFAA), pentafluoropropionic anhydride (PFPA), and heptafluorobutyric anhydride (HFBA) have been used because they facilitate rapid, complete, one step derivatization reactions for the biogenic amines and their metabolites[64-69].

In this work, TFAA was mainly used and reactions with it proved to be very handy, rapid and complete. The trifluoroacetyl derivatives of several biogenic amines and related compounds are listed in Table 3.1. The fluorinated acylating reagent can completely acylate the active hydrogens of most biogenic amines and metabolites used in the present experiments. Even though the products are simple for most cases, the number and proportions of the products of NE, 5-HT and TRA were found to vary according to as yet undetermined factors. When NE was reacted with TFAA in the presence of ethyl acetate at room temperature for 1 hour, there were three different products; TFA_4-NE, cis and trans TFA_3-NE.

TABLE 3.1.

Trifluoroacetyl derivatives of several biogenic amines and DHPG, and their molecular weights.

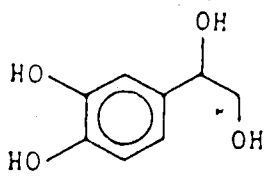
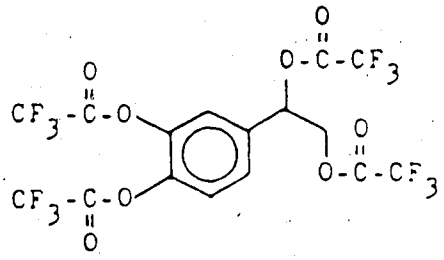
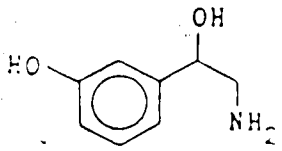
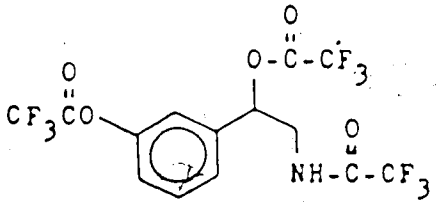
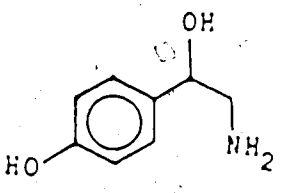
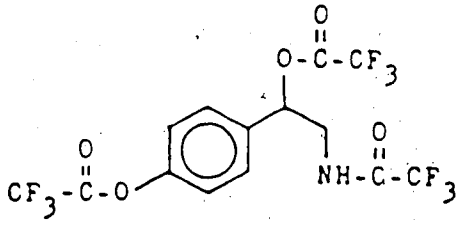
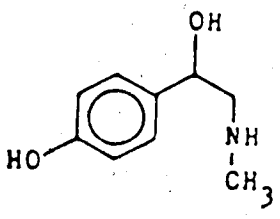
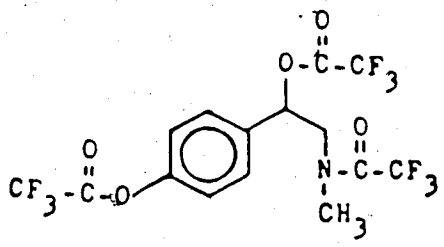
COMPOUND	M.W.	TFA-DERIVATIVE	M.W.
 DHPG	170.2	 TFA_4-DHPG	554.0
 M-OCT	153.2	 TFA_3-M-OCT	441.0
 P-OCT	153.2	 TFA_3-P-OCT	441.0
 SYNE	167.2	 TFA_3-SYNE	455.0

TABLE 3.1 (continued)

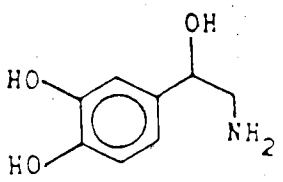
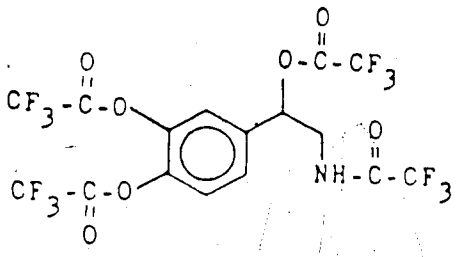
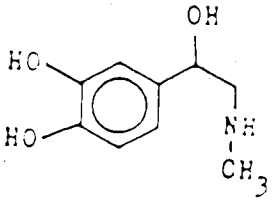
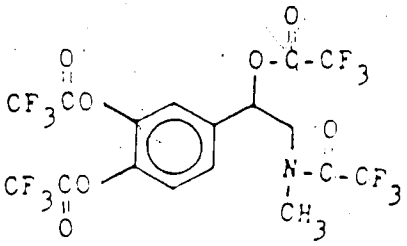
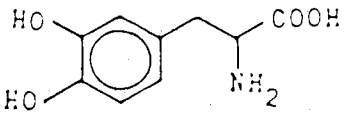
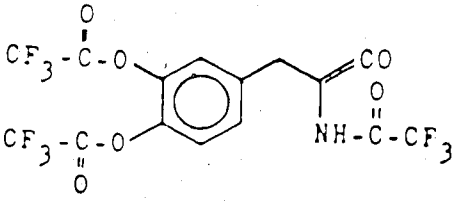
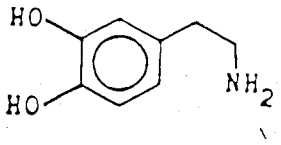
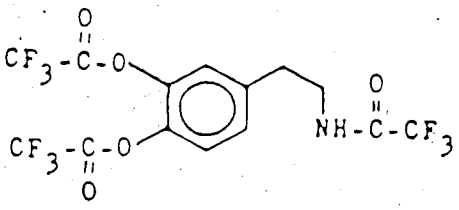
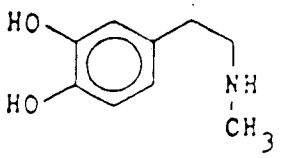
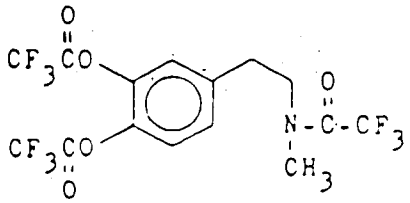
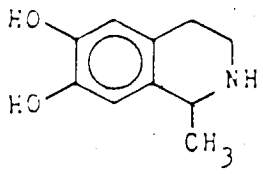
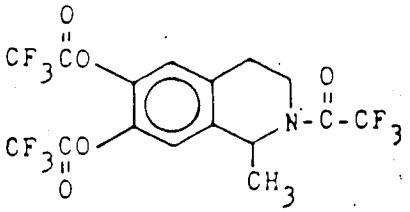
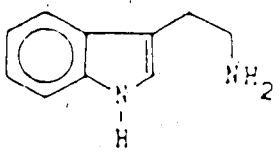
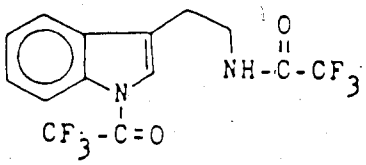
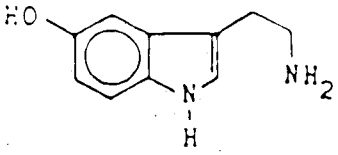
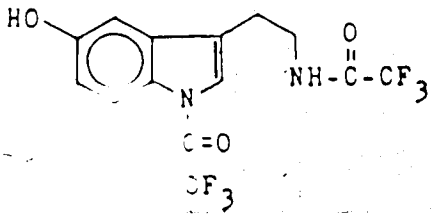
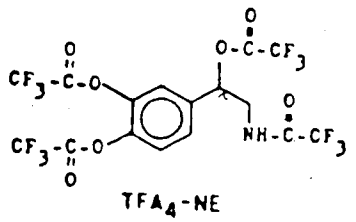
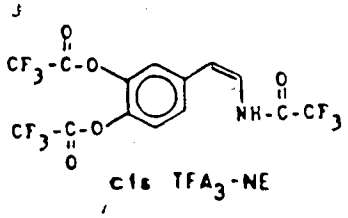
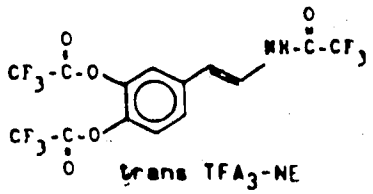
COMPOUND	M.W.	TFA-DERIVATIVE	M.W.
 NE	169.2	 TFA_4-NE	553.0
 EN	183.2	 TFA_4-EN	567.0
 DOPA	197.2	 TFA_3-DOPA	467.0
 DA	153.2	 TFA_3-DA	441.0

TABLE 3.1.(continued)

COMPOUND	M.W.	TFA-DERIVATIVE	M.W.
 NMDA	167.2	 TFA_3-NMDA	455.0
 SALS	179.2	 TFA_3-SALS	467.0
 TRA	160.2	 **TFA_2-TRA**	**352.1**
 5-HT	176.2	 TFA_2-5-HT	368.1







The presence of the two TFA_3-NE isomers was deduced from their retention times in the gas chromatograph and similar mass spectrometric fragmantation patterns. These cis and trans isomers seem to be formed by elimination of trifluoroacetic acid from the side chain of the TFA_4-NE where all acidic hydrogens were replaced with trifluoroacetyl group. When TRA and 5-HT were derivatized with TFAA, more than 3 products were formed. But some of them seemed to be very unstable at room temperature. Within several hours after the products were left standing at room temperature, the intensities of these products varied a lot in the total ion current chromatograms. The major derivatives of TRA and 5-HT are shown in Table 3.1.

3.2. THE MASS SPECTRA OF BIOGENIC AMINES AND DHPG

The NICI and ammonia PCI spectra of TFA derivatives of several biogenic amines and metabolites are shown in Fig. 3.1 to Fig. 3.13. Because there are too many peaks in the ammonia PCI,

only the peaks whose relative intensities are greater than 5%, are shown in the Figures. Several characteristic fragmentations in each ionization mode are observed and classified in Table 3.2 to Table 3.4. The characteristic fragmentations will be discussed in the section, "Characteristic Fragmentations". In the section, "Individual Spectra", the spectra of compounds used in the present study are shown and the fragmentation patterns are examined for each ionization mode.

3.2.1. CHARACTERISTIC FRAGMENTATIONS

In NICI, the molecular ions are not prominent (Table 3.2), but the base peak was usually greater than 50% of the total ion current. The base peak is formed by elimination of a CF_3CO (TFA) group from the molecular anion. Wood[30] has observed similar loss and has suggested that the loss of the derivatizing group is probably from the catechol rather than the amine position in the study of NICI spectra of PFP derivatives of DA and several biogenic amine metabolites; however, no plausible evidence was given for this[30].

As shown in Table 3.2, the catechol compounds show significant amounts of the $[M-TFA]^-$ ion while indole compounds and phenolic amines do not. Therefore, it is deducible that the elimination of a TFA group is related with the catechol structure and comes from the phenolic position which is in

TABLE 3.2
MAJOR IONS OF BIOGENIC AMINES AND DHPG WHEN METHANE WAS USED AS THE REAGENT GAS IN THE NEGATIVE ION CHEMICAL IONIZATION MODE(NICI)

TFA DERIVATIVE	MAJOR IONS (Values are relative intensities %)					
	M^-	$[M-HF]^-$	$[M-TFA]^-$	$[M-TFAOH]^-$	$[M-HF-TFA]^-$	$[M-TFA-TFAOH]^-$
DHPG	-	-	63.3	-	-	100
M-OCT	1.7	6.7	3.0	100	-	-
P-OCT	1.7	1.5	6.7	100	-	6.0
SYNE	-	40.0	6.8	-	100	-
NE	10.7	-	67.4	4.3	-	100
EN	1.6	-	100	-	-	39.0
DOPA	8.5	-	100	8.5	-	-
DA	7.5	-	100	-	-	-
NMDA	-	-	100	-	-	-
SALS	-	-	100	-	-	-
TRA	3.3	100	-	-	-	-
5-HT	-	100	-	-	-	-

agreement with Wood's suggestion.

The $[M-TFA]^-$ fragment is amongst the most useful for the mass spectrometric identifications. The $[M-HF]^-$, $[M-TFAOH]^-$, and other ions of Table 3.2 occur only for some compounds and will be discussed in the individual spectra section.

Many of the derivatized compounds had a prominent peak at mass unit 113, which corresponds to CF_3COO^-. The CF_3COO^- ion must be formed by cleavage of the phenolic C-O bond. Also observed were the OH^- adducts and CF_3COO^- adducts to the molecule, M, when the pressure of the ion source was high. These ions could be useful for identifying the molecular weight of M. Dougherty et al[70] observed $[M+Cl]^-$ ion in NICI spectra of polycyclic chlorinated insecticides. They thought that the ions, $[M+Cl]^-$, occur by a clustering reaction between the sample molecule, M, and Cl^-, which is the primary product of the ionization. The present results are somewhat analogous, but in this case a large group, CF_3COO^-, instead of a single atom attaches itself to the neutral molecule to form a negative ion, $[M+CF_3COO]^-$.

When positive chemical ionization was employed with ammonia as the reagent gas, the trifluoroacetyl derivatives of several biogenic amines had the ion-molecule adducts $[M+NH_4]^+$ as base peaks (Table 3.3). Because the most abundant ion was

TABLE 3.3
MAJOR IONS OF BIOGENIC AMINES AND DHPG WHEN AMMONIA WAS USED AS THE REAGENT GAS IN THE POSITIVE CHEMICAL IONIZATION MODE(PCI)

TFA DERIVATIVE	MAJOR IONS (Values are relative intensities %)[a]				
	$[M+NH_4]^+$	M^+	$[M+NH_4-TFA+H]^+$	$[M+NH_4-TFAO+H]^+$	$[M+NH_4-TFA-TFAO+2H]^+$
M-OCT	100	-	6.7	33.2	13.3
P-OCT	100	-	-	50.0	41.7
SYNE	100	-	-	46.7	21.7
EN	100	-	-	21.7	32.0
DOPA	100	-	6.3	-	-
DA	100	-	13.3	8.3	6.3
NMDA	100	-	10.0	10.0	-
SALS	100	-	50.0	18.3	41.7
TRA	100	19.4	33.3	24.6	-
5-HT	100	6.7	-	-	-

[a] Relative intensities less than 5% are excluded.

$[M+NH_4]^+$ in all cases, the spectra were very simple. Therefore, this ionization mode provides unequivocal information on the molecular weight of the analyte. The trifluoroacetyl derivative of DHPG was not detected in ammonia PCI. One of peculiar things of the present experimental observation was that no $[MH]^+$ existed in the spectra. It can be suggested that the proton affinities of the derivatized biogenic amines are lower than that of ammonia, so no proton transfer reaction can occur from the ammonium ion to the sample molecule. It is known that strong electron withdrawing groups like CF_3CO when attached to nitrogen lead to a substantial reduction of the nitrogen basicity. More detailed information on the relation between the proton affinity and proton transfer reation can be found in references[34, 71-72].

Miyazaki et al[24] have previously studied the ammonia PCI of PFP derivatives of biogenic amines and their metabolites. They observed no elimination of derivatizing reagent specific groups in the spectra[24]. On the contrary, in the present study the elimination of TFA or TFAO groups from the TFA derivatized biogenic amines is observed, see Table 3.3. However the peaks correspond to loss of TFA or TFAO with a simultaneous pickup of one H atom. It is of interest to find out where the hydrogen atom comes from. A similar process was also observed in the methane PCI where elimination of the TFA and TFAO groups from $[M+H]^+$ ions and a pickup of a hydrogen atom occurs. Stemmler and Hites[73] recently repc d the NICI spectra of 24

hexachlorocyclopentadiene derivatives where they observed unusual prominent ion of type, $[M+nH-nCl]^-$ or series of ions differing by 34 mass units, $[+nH-nCl]$, where n=1 to 4 [73]. Grimsrud et al[74] also observed M^-, $[M+H-X]^-$, $[M-2X]^-$, and $[M+H-X]^-$ in the NICI spectra of fluoranil and chloranil where methane was used as a reagent gas. Taking EI spectra of fluoranil after subjecting the ion source to NICI conditions for different time intervals, they observed that the intensity of $[M+2H]^+$ increased according to the time for which the ion source was subjected to the NICI condition. They obtained also evidence that an efficient wall reaction occurs in the ion source. They have suggested that H atoms adsorbed on the ion source walls hydrogenate the neutral sample molecules and that activation(hydrogen loading) of the ion source walls is caused by the intense electron irradiation of methane under the normal NICI conditions[74]. The phenomena observed in the present work in the ammonia and methane PCI, also suggest that the wall hydrogenation reaction occurs under PCI conditions. The hydrogenated MH on forming an adduct with NH_4^+ undergoes then a simple loss of TFA or TFAO.

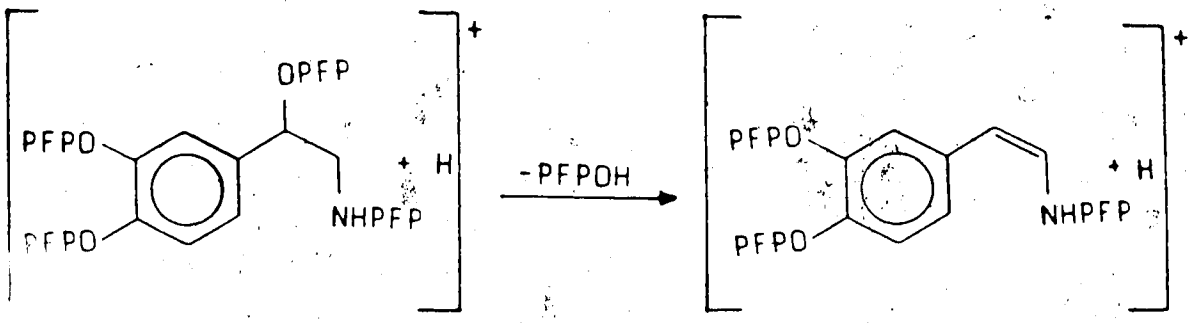
When methane was used as the reagent gas for the positive chemical ionization, $[M+H]^+$ ions were recorded as the base peaks of derivatized DA, NMDA, SALS, TRA and 5-HT (Table 3.4). The elimination of pentafluoropropionic acid(PFPOH) from the PFP derivative of norepinephrine was observed[24] in the methane PCI and the following mechanism has been suggested[24].

TABLE 3.4
MAJOR IONS OF BIOGENIC AMINES AND DHPG WHEN METHANE WAS USED AS THE REAGENT GAS IN THE POSITIVE CHEMICAL IONIZATION MODE(PCI)

TFA DERIVATIVE	MAJOR IONS (Values are relative intensities %)[a]				
	MH^+	$[MH-TFA+H]^+$	$[MH-TFAO+H]^+$	$[MH-TFANH_2]^+$	$[MH-TFAOH]^+$
DHPG	-	-	-	-	100
M-OCT	-	-	22.8	-	100
P-OCT	-	-	55.3	-	100
SYNE	5.0	-	15.1	-	100
EN	5.3	-	20.9	-	100
DA	100	13.3	11.7	-	21.7
NMDA	100	13.4	11.0	-	28.9
SALS	100	35.0	16.7	-	-
TRA	100	91.9	-	63.3	-
5-HT	100	-	-	21.7	-

[a] Relative intensities less than 5% are excluded.

[Scheme I]



In the present work, the loss of mass 114, corresponding to TFAOH, was observed in the derivatized products of several biogenic amines and DHPG. The above fragmentation type probably occurs, provided that there is a hydroxyl group in the alkyl chain of the molecular structure. The fragment ions formed in this fragmentation were the base peaks of the TFA derivatives of DHPG, M-OCT, P-OCT, SYNE and EN.

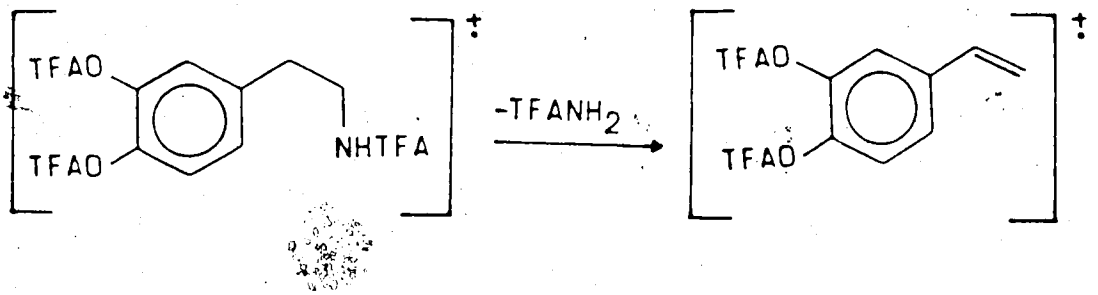
In Electron Impact(EI), fragmentation is significant and molecular ions are generally not observed. Only DOPA shows a significant amount of the molecular ion(Table 3.5). Several major ions and relative intensities of biogenic amines and DHPG are listed in Table 3.5. Table 3.5 shows that one of the prominent fragmentations in the spectra of TFA derivatives of several biogenic amines(M-OCT, SYNE, EN, and DA) is cleavage of the bond between alpha and beta carbon atoms of the alkyl chain with retention of the positive charge mostly on the nitrogen containing fragment, leaving an ion at m/e=126,

TABLE 3.5
Several fragment ions of biogenic amines and metabolite in the electron impact (EI) ionization mode

TFA DERIVATIVE	FRAGMENT IONS (Values are relative intensities %
DHPG	440(100) 343(15.0) 327(16.7) 287(20.0) 246(30.0) 229(26.7) 133(26.8) 97(16.7) 69(43.3)
M-OCT	328(58.3) 315(50.0) 287(26.7) 217(11.7) 126(100) 105(31.7) 69(58.3)
P-OCT	328(33.3) 315(46.7) 287(18.3) 217(8.3) 126(28.3) 69(100)
SYNE	342(13.3) 328(23.0) 315(12.0) 287(10.0) 245(10.0) 217(17.0) 140(100) 110(33.7) 69(95.3)
EN	454(10.8) 440(9.2) 357(4.2) 140(100) 69(73.3)
DOPA	467(43.3) 341(31.7) 315(25.0) 287(24.7) 259(10.3) 216(22.0) 69(100)
DA	328(100) 287(11.7) 231(13.3) 126(61.7) 69(56.7)
NMDA	341(40.0) 327(70.0) 230(25.0) 214(16.7) 130(18.3) 116(23.3) 110(100)
SALS	467(14.2) 452(100) 69(13.3)
TRA	352(16.7) 239(100) 226(93.3) 129(31.7) 69(18.3)
5-HT	368(10.0) 255(28.3) 242(100) 145(18.3) 117(15.0) 69(8.3)

$[CF_3CONHCH_2]^+$, or an ion at m/e=140 $[CF_3CON(CH_3)CH_2]^+$, if there is a methyl substituent on the nitrogen. This fragmentation was observed previously[75] in the spectra of TFA derivatives of p-hydroxyamphetamine and p-hydroxynorephedrine and the above explanation has been suggested by Belvedere. Another major fragmentation in the TFA derivatives of M-OCT, P-OCT, SYNE, EN, DA, NMDA, TRA, and 5-HT is a mass loss of 113 which may correspond to either CF_3CONH_2 or CF_3COO. Belvedere[75] suggested that this loss is due to the elimination of CF_3COO from the benzene ring rather than the alkyl chain. On the contrary, Duncan[64] suggested that CF_3CONH_2 is eliminated by a McLafferty rearrangement as shown below for the case of a TFA derivatized dopamine.

[Scheme II]



3.2.2. INDIVIDUAL SPECTRA

The dopamine derivative spectrum in NICI is shown in Fig 3.1 (a). The small peak, whose m/e is 441, is the molecular anion. In NICI, molecular ions did not predominate. There is also a $[M-20]^-$ ion which is due to loss of HF from the molecular

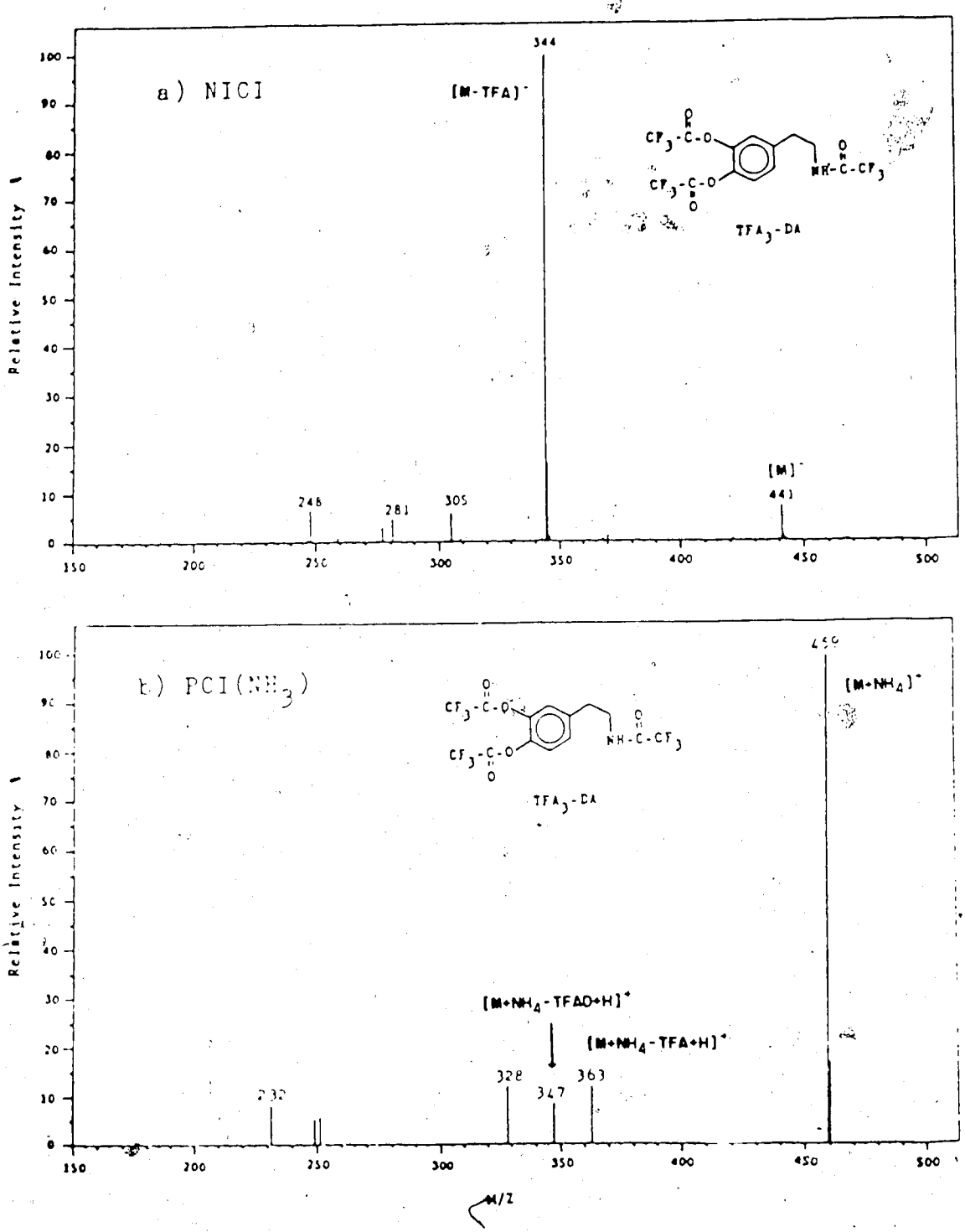


Figure 3.1 a). NICI spectrum of TFA derivative of dopamine.
b). PCI(ammonia) spectrum of TFA derivative of dopamine.

anion. Even though the intensity of this peak is very weak in the TFA derivative of DA, the elimination of HF from the PFP derivative is a major fragmentation pathway and $[M-HF]^-$ ion is the base peak[28]. The biggest peak in the spectrum of the TFA derivative of dopamine is an ion at m/e=344 which comes from loss of a TFA group from the catechol derivatives. This base peak was usually greater than 60 % of the total ion current. The successive loss of a TFA group from the ion at m/e=344 results in an ion at m/e=248.

In ammonia PCI, the base peak of the dopamine derivative mass spectrum is an ion at m/e=459 which is $[M+NH_4]^+$ (Fig. 3.1 (b)). This peak shows that the molecular mass of the dopamine derivative is 441. Other characteristic fragment ions of the dopamine derivative in ammonia PCI are listed in Table 3.3. The ion at m/e=363 comes from elimination of a TFA group and a pick up of a hydrogen atom from the ion source walls.

In Fig 3.2, there are EI and methane PCI mass spectra of the dopamine derivative. In EI spectrum, there are a lot of fragment ions as expected and the base peak, an ion at m/e=328, comes from loss of mass 113 corresponding to either CF_3COO or CF_3CONH_2.

The base peak is an ion at m/e=442 which is $[M+H]^+$ in methane PCI. An ion at m/e=346 comes from loss of a TFA group from the pseudo molecular ion $[M+H]^+$ and pick up of a hydrogen atom. The ion at m/e=328 comes from loss of trifluoroacetic acid

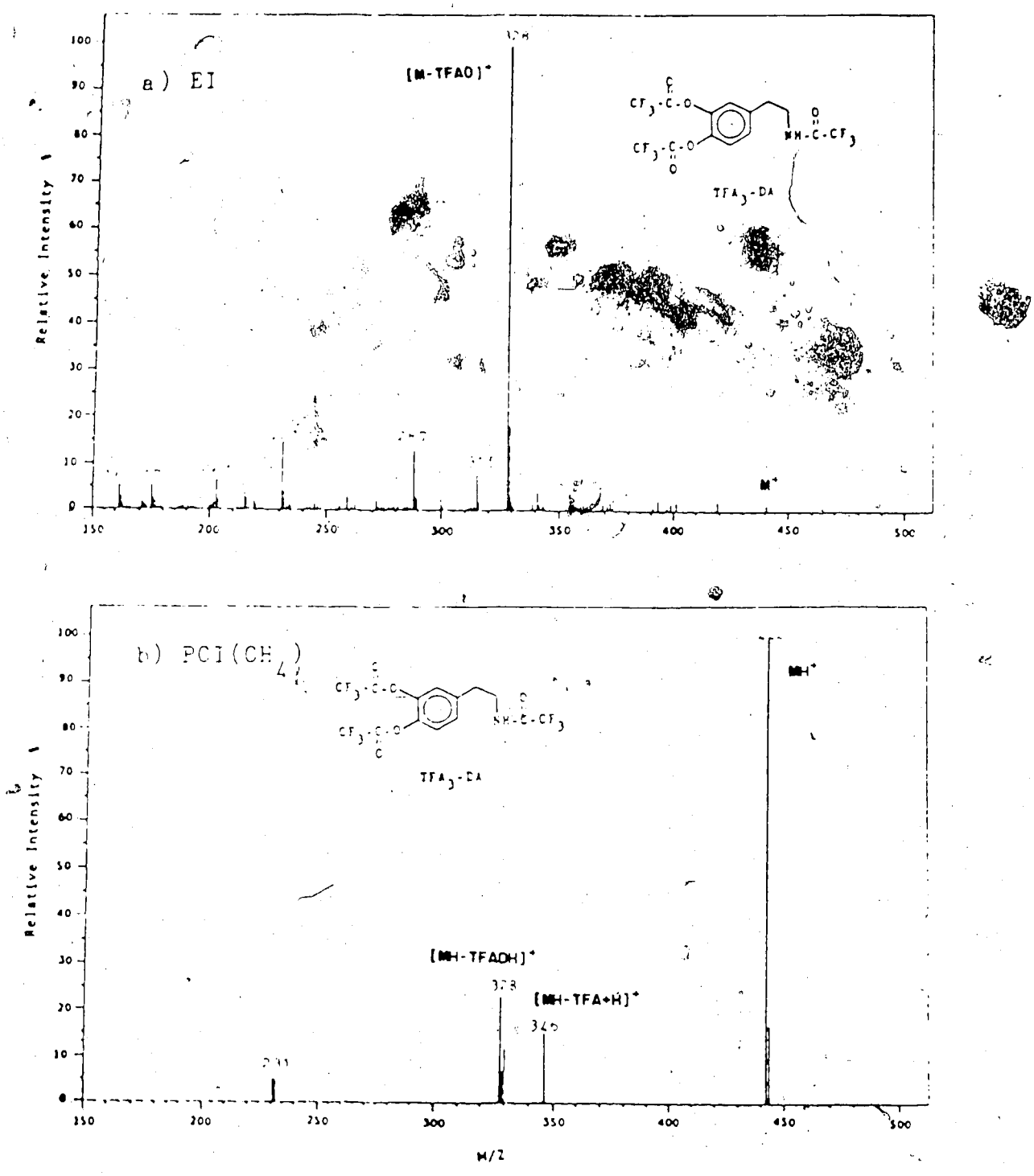
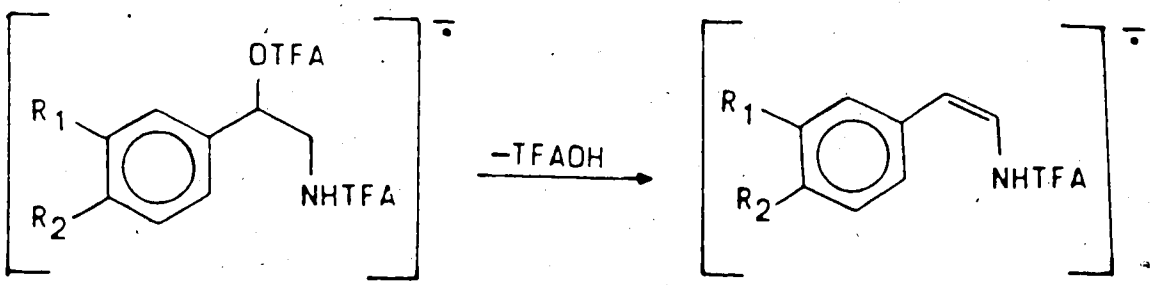


Figure 3.2 a). EI spectrum of TFA derivative of dopamine.
b). PCI(methane) spectrum of TFA derivative of dopamine.

from the ion at 442. The other fragment ions are classified and listed in Table 3.4.

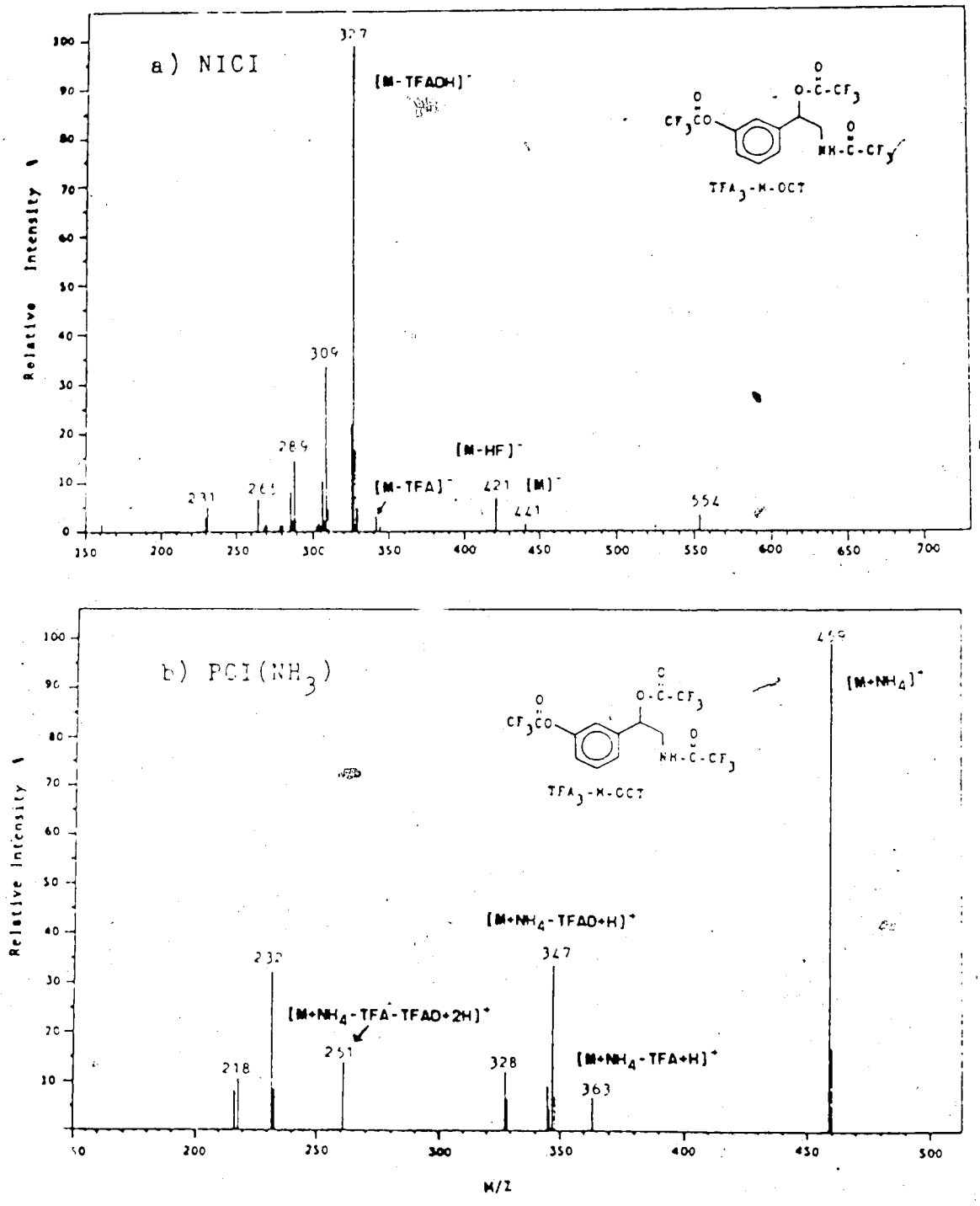
In contrast with the spectra of the dopamine TFA derivative, the spectra of two isomers of dopamine, meta and para octopamine derivatives, show significant differences in their fragmentation patterns (Fig. 3.3 and 3.4, respectively). The base peak ions of meta and para octopamine derivatives at m/e=327, comes from loss of mass 114, corresponding to the mass of trifluoroacetic acid, from the molecular anion. By inspecting the molecular structures of TFA derivatives of meta and para octopamine, it can be suggested that the trifluoroacetic acid is eliminated from the alkyl chain of the molecular anion as shown in the following scheme:

[Scheme III]



	R_1	R_2
M-OCT	TFAO	H
P-OCT	H	TFAO

Duffield[76] reported different fragmentation processes in the study of NICI spectra of deuterated analogues of ortho-, meta-,



gure 3.3 a). NICI spectrum of TFA derivative of M-OCT.
b). PCI(ammonia) spectrum of TFA derivative of M-OCT

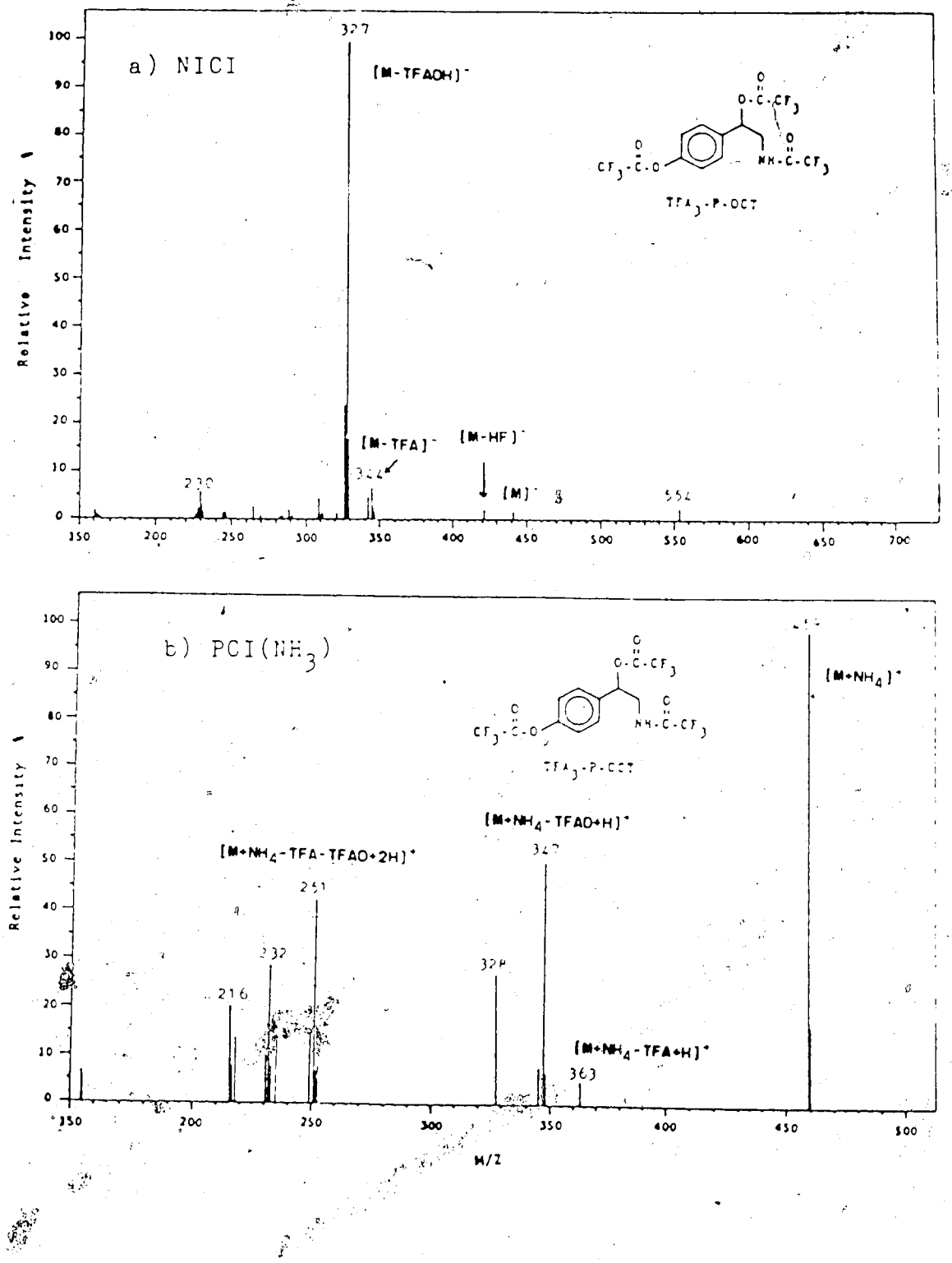


Figure 3.4 a). NICI spectrum of TFA derivative of P-OCT.
b). PCI(ammonia) spectrum of TFA derivative of P-OCT.

and para-tyramine-N,O-dipentafluoropropionic derivatives. He observed that the base peak ions of PFP derivatives of meta and para tyramine are different. He proposed that the base peak ion of the PFP derivative of the para tyramine, $[M-H-PFP]^-$, is formed by elimination of PFP from the phenol derivative and a hydrogen atom from the nitrogen atom of the molecular anion. However, it was observed that an ion at 327 is the base peak in both spectra of the TFA derivatives of meta and para octopamine and an ion at m/e=343, corresponding to $[M-H-TFA]^-$, does not exist in the spectra of the TFA derivatives of meta and para octopamine. Therefore, the fragmentation patterns observed by Duffield do not occur in the NICI spectra of the TFA derivatives of meta and para octopamine. The main reason of the difference in the fragmenatation patterns between tyramine and octopamine comes from the fact that octopamine has a hydroxyl group in the alkyl chain while tyramine does not.

The NICI and ammonia PCI spectra of two isomers, N-methyldopamine(NMDA) and synephrine(SYNE) TFA derivatives, are shown in Fig. 3.5 and Fig. 3.6, respectively. The molecular weight of each derivative is 455 (Table 3.1) and it can be derived from the ammonia PCI spectra where the base peak is an ion at 473 corresponding to $[M+NH_4]^+$. It is clearly shown that the fragmentation patterns in the NICI spectra of NMDA and SYNE are very different. The base peak of NMDA derivative comes from elimination of a TFA group from the molecular anion in NICI and this base peak ion seems to be very stable so the NICI

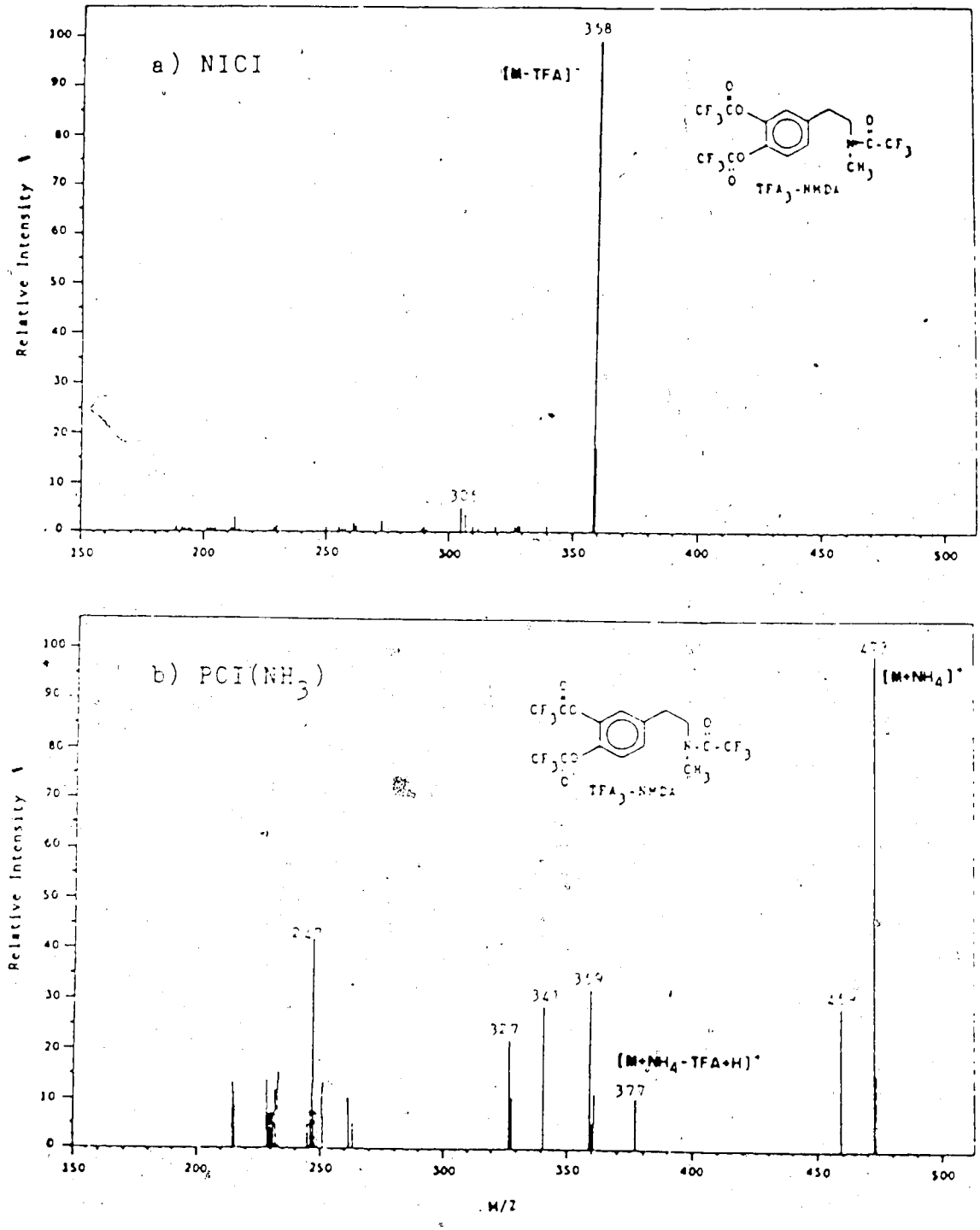


Figure 3.5 a). NICI spectrum of TFA derivative of NMDA.
b). PCI(ammonia) spectrum of TFA derivative of NMDA.

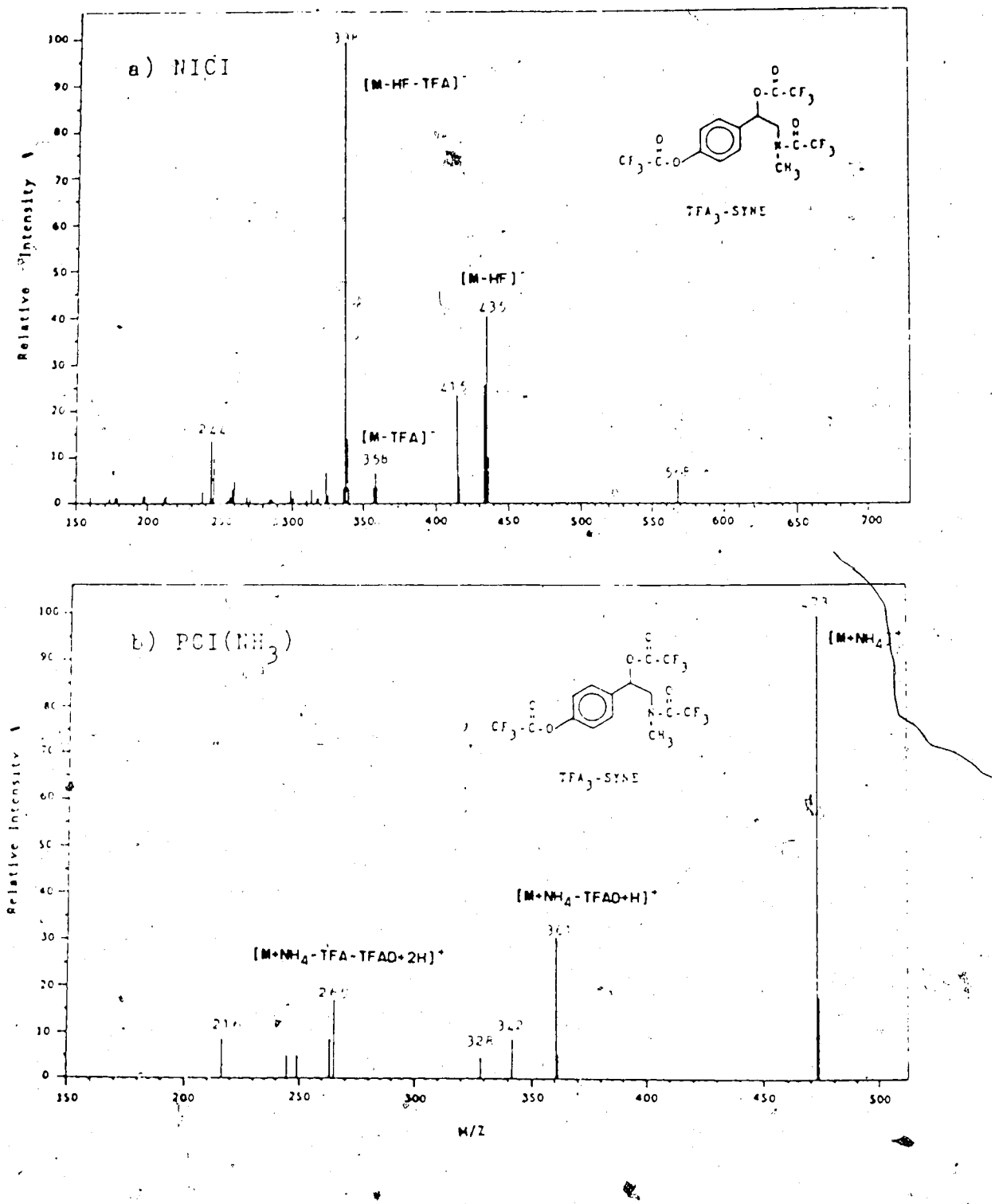


Figure 3.6 a). NICI spectrum of TFA derivative of SYNE.
b). PCI(ammonia) spectrum of TFA derivative of SYNE.

spectrum is much simpler. On the contrary, in the fragmentation of SYNE derivative in NICI, there is $[M-HF]^-$ peak at m/e=435. The base peak at m/e=338, comes from the elimination of a TFA group from the $[M-HF]^-$ ion.

There are also a lot of fragmentation differences in the ammonia PCI. In the NMDA spectrum, there is an ion at m/e=459 which comes from elimination of a methyl group from the side chain and pickup of a hydrogen atom, and the ion is relatively strong in intensity. But in the SYNE derivative spectrum, there is no initial fragmentation of the methyl group and the rest of the fragment ions do not correspond to those of the NMDA derivative.

The methane PCI spectrum of the NMDA derivative is similar in fragmentation to that of the dopamine derivative and the spectrum of the SYNE derivative is similar to that of the octopamine derivative (see Table 3.4). This similarity in fragmentation depends on whether there is a hydroxyl group in the side chain, or not.

The NICI spectra of NE, EN, and DHPG (Fig. 3.7 and Fig. 3.8) also gave interesting results. NE, EN, and DHPG have a catechol group and a hydroxyl group in the alkyl chain of the molecule. It is of interest to find out which fragmentation process occurs first; the elimination of the catechol derivative or the hydroxyl derivative. No $[M-TFAOH]^-$ ion exists in the spectra of these three compounds (m/e=439, 453, and 440 for NE, EN, and

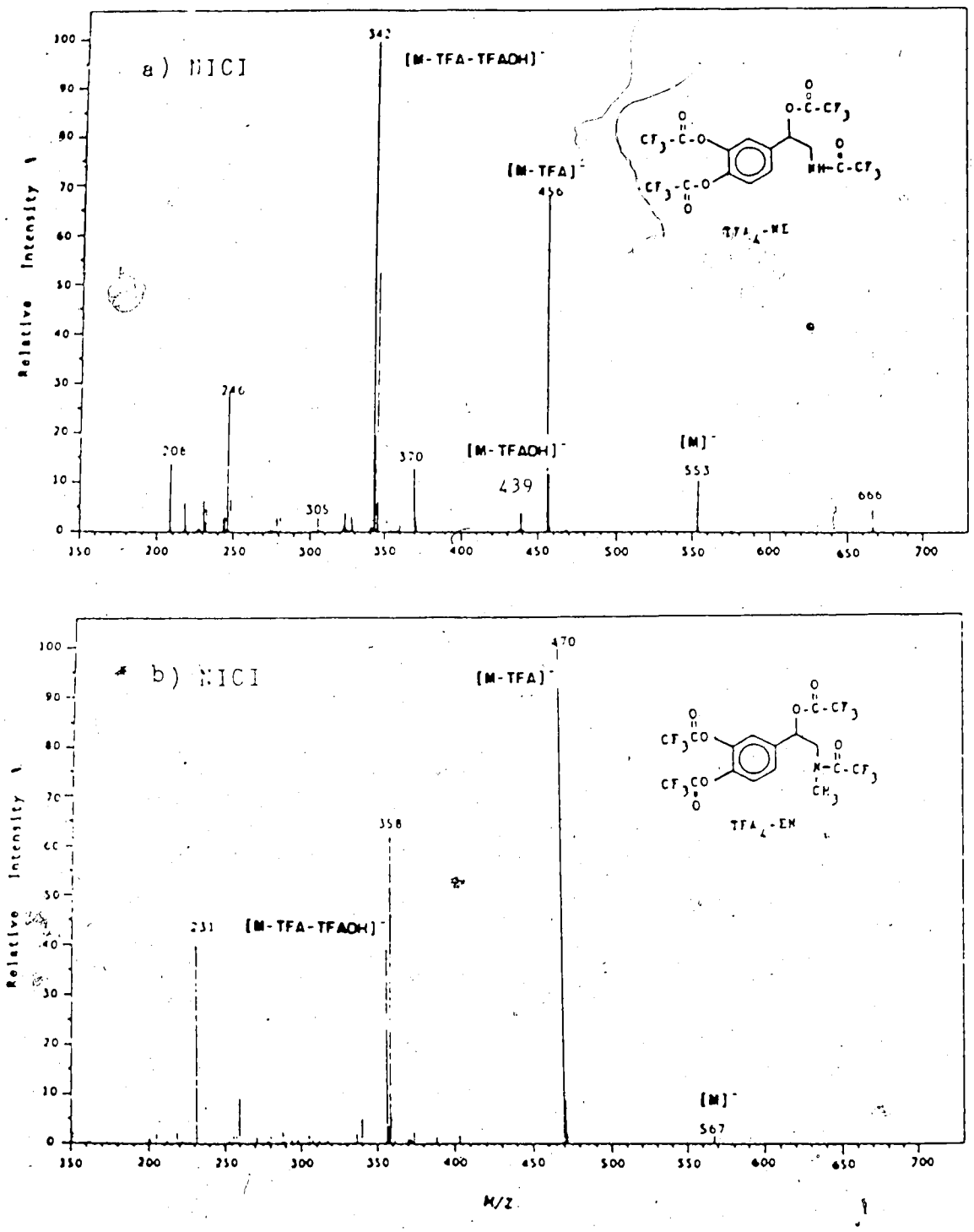


Figure 3.7 a). NICI spectrum of TFA derivative of NE.
b). NICI spectrum of TFA derivative of EN.

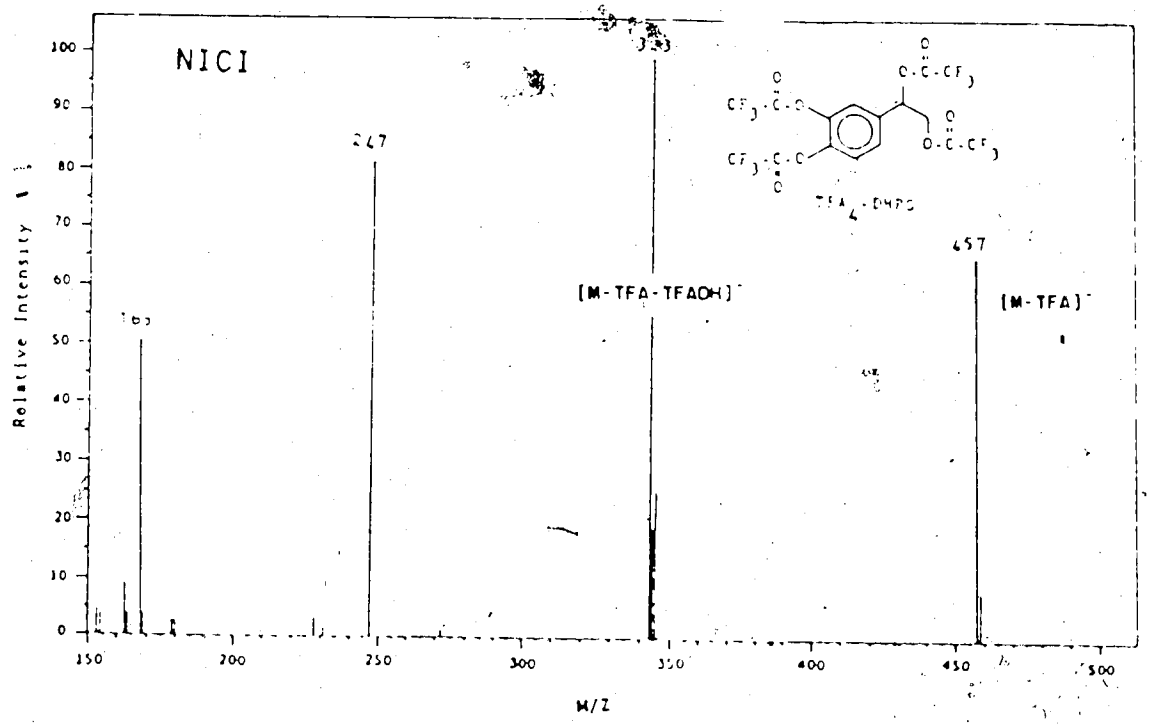
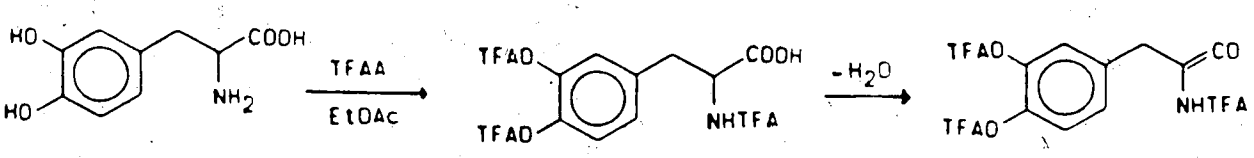


Figure 3.8 NICI spectrum of TFA derivative of DHPG.

DHPG, respectively) except NE (less than 5%). If the elimination of trifluoroacetic acid occurred first, the above ions should exist in a significant amount. The $[M-TFA]^-$ ion exists at 456, 470, and 457 (more than 60 % relative intensity) for NE, EN, and DHPG, respectively. The $[M-TFA-TFAOH]^-$ ion exists also as a base peak for DHPG and EN. The precusor of the ion, $[M-TFA-TFAOH]^-$, must be the ion, $[M-TFA]^-$. From the above results, it can be suggested that the elimination of a catechol derivative occurs first followed by the elimination of the hydroxyl derivative in the alkyl chain.

The NICI spectrum of TFA derivatized DOPA is shown in Fig. 3.9 (a). The expected molecular weight of this derivatized product is 485. But there is not an ion of this mass in the spectrum. The highest mass anion of the DOPA TFA derivative occurs at m/e=467. This mass corresponds to the loss of one mole of water from the supposed structure(X). In the same run, an ion at m/e=484, was observed which corresponds in mass to the adduct cluster of OH^- to the sample molecule.



DOPA (X)

The base peak of the NICI spectrum of DOPA derivative is an ion at m/e=370 which comes from loss of a TFA group from the highest

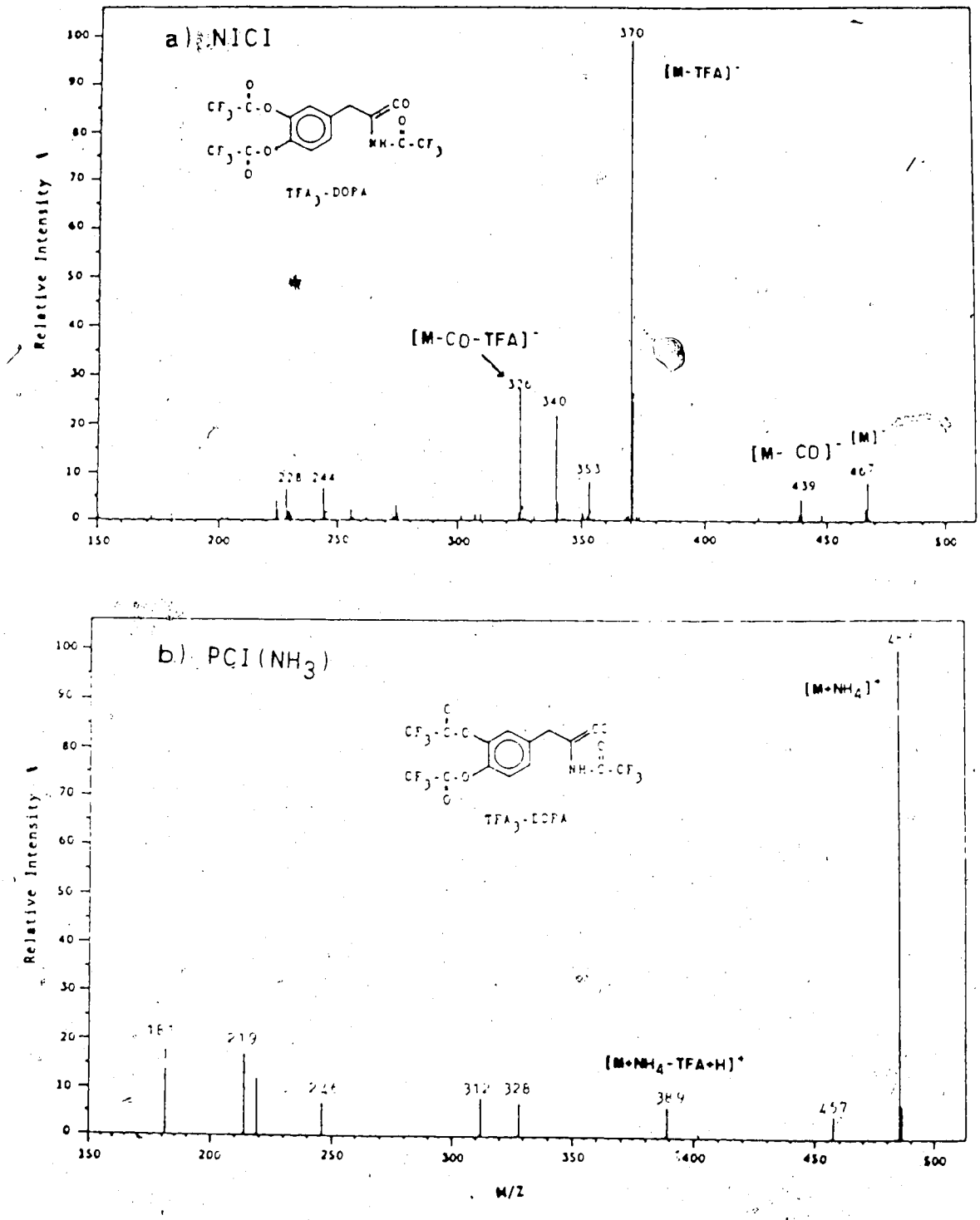


Figure 3.9 a). NICI spectrum of TFA derivative of DOPA.

b). PCI(ammonia) spectrum of TFA derivative of DOPA.

mass anion. There is also an ion at m/e=439 which is due to loss of CO from the highest mass anion. This peak strongly suggests that the product of the derivatizing reaction has a CO functional group in the molecular structure.

The base peak in the ammonia PCI spectrum is a $[M+NH_4]^+$ ion at 485 (Fig. 3.9 (b)). This data shows that the molecular mass of the TFA derivative of DOPA is 467, and excludes the possibility of a dehydration reaction in the ion source.

The EI spectrum of the derivative of DOPA is shown in Fig. 3.10. Even though the intensity of an ion at 439 is low, this ion comes from loss of CO from the molecular ion at 467. EI spectrum also suggests the derivatized product has a CO functional group. Therefore, there is a dehydration reaction when DOPA is derivatized with TFAA in ethyl acetate.

The NICI spectrum of derivatized salsolinol(SALS) shows only one big base peak at m/e=370 which comes from loss of a TFA group from the molecular anion(m/e=467). The molecular anion is not present in the spectrum (Fig. 3.11). The ammonia PCI shows that the molecular mass is 467 because there is a base peak at m/e=485 which is a $[M+NH_4]^+$ (Fig. 3.11(b)). The EI spectrum of SALS shows that the base peak ion comes from elimination of a methyl group from the molecular ion (Fig. 3.12). This behaviour of TFA derivative of SALS in EI was already observed[64]. This behaviour can be explained since a stable secondary carbonium ion is formed by losing the methyl group[77].

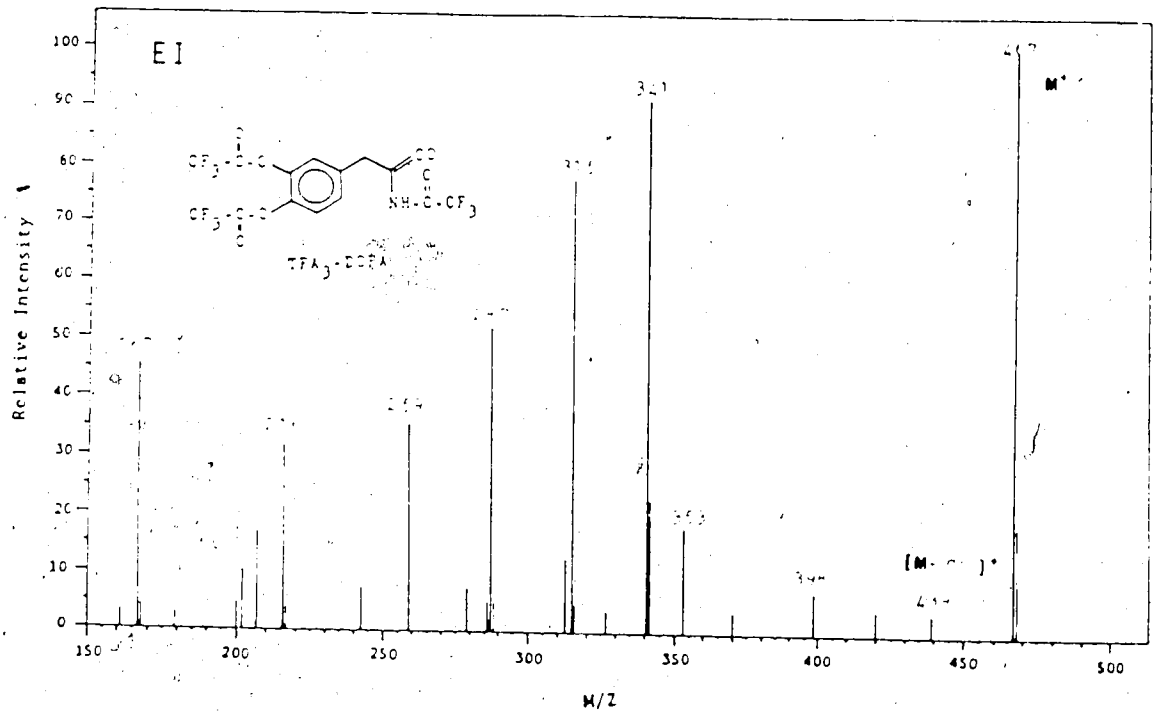


Figure 3.10 EI spectrum of TFA derivative of DOPA.

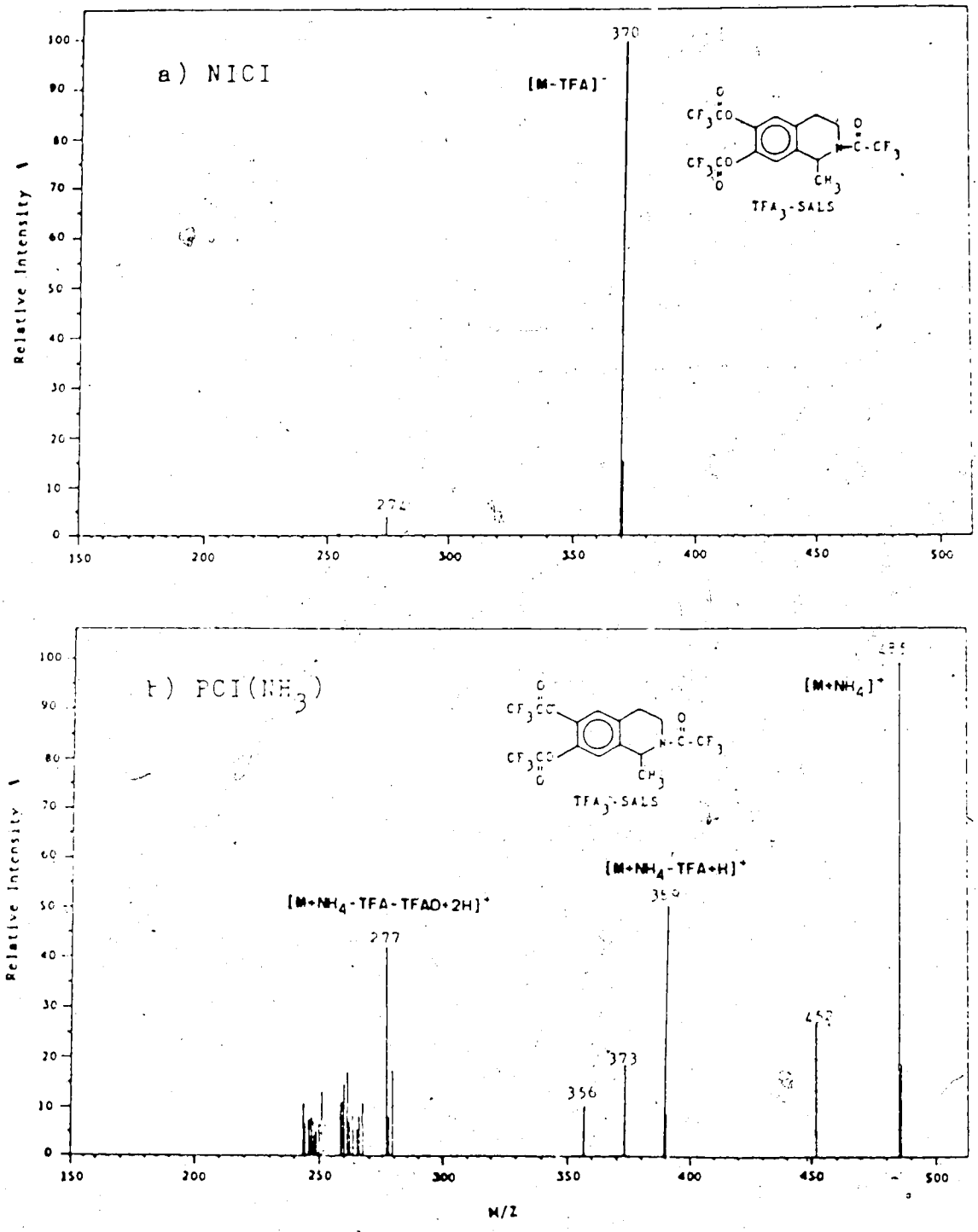


Figure 3.11 a). NICI spectrum of TFA derivative of SALS.

b). PCI(ammonia) spectrum of TFA derivative of SALS.

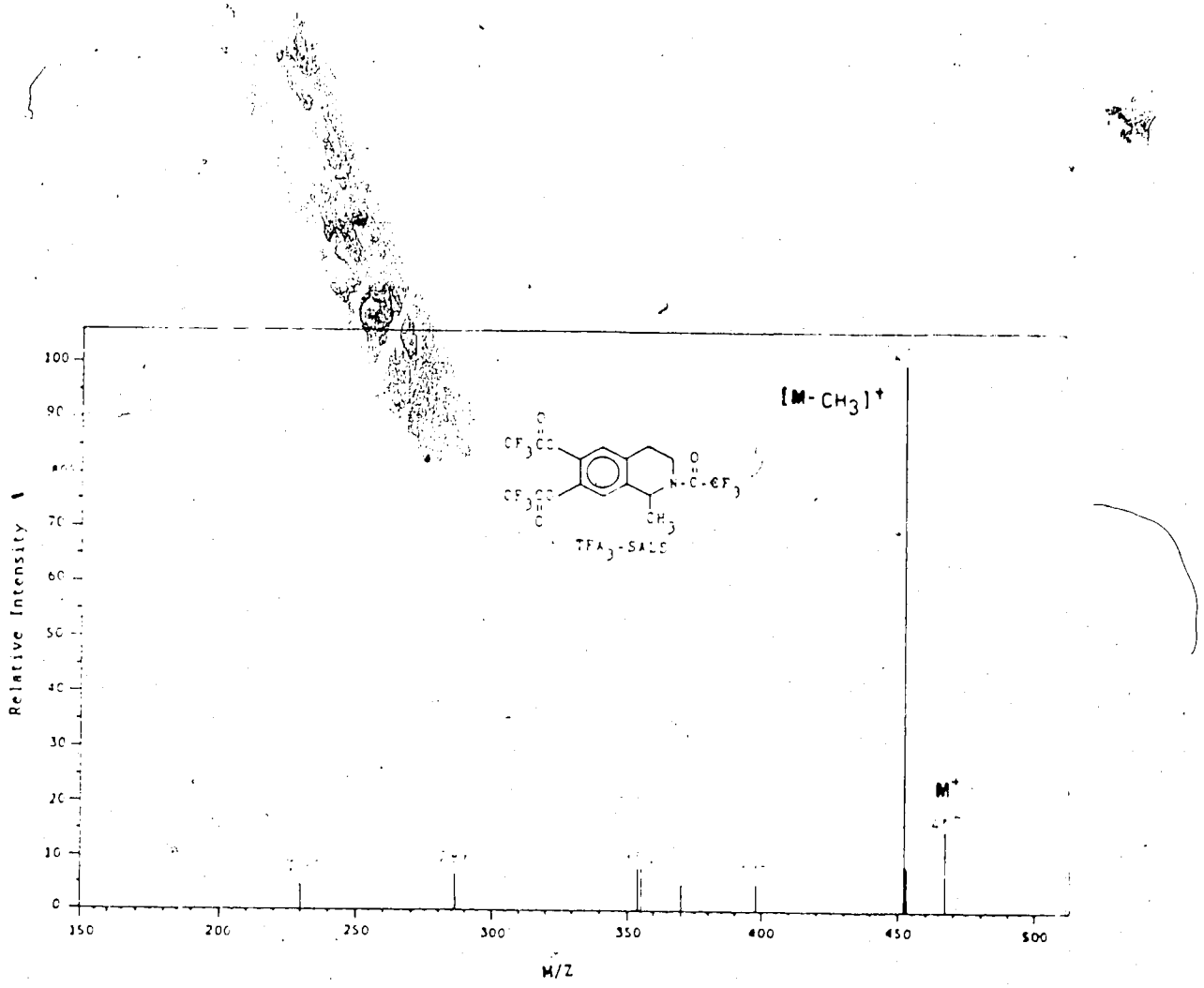


Figure 3.12 EI spectrum of TFA derivative of SALS.

The NICI and ammonia PCI spectra of TFA derivatized tryptamine(TRA) and 5-hydroxytryptamine (5-HT) are shown in Fig. 3.13 and Fig. 3.14. The base peaks of these products are due to the of loss of HF from the molecular anions. Even though no molecular anion is shown in 5-HT, the ammonia PCI sho s that the molecular weight of the derivatized product is 368. nd in these compounds, there are M^+ ions in the ammonia PC An ion at m/e=328 in NICI spectrum of 5-HT derivative comes om loss of 2 moles of HF from the molecular anion which is not shown in the spectrum. The ion at m/e=304 comes from elimination of H_2NCO radical from the base peak of NICI spectrum of TFA derivative of 5-HT.

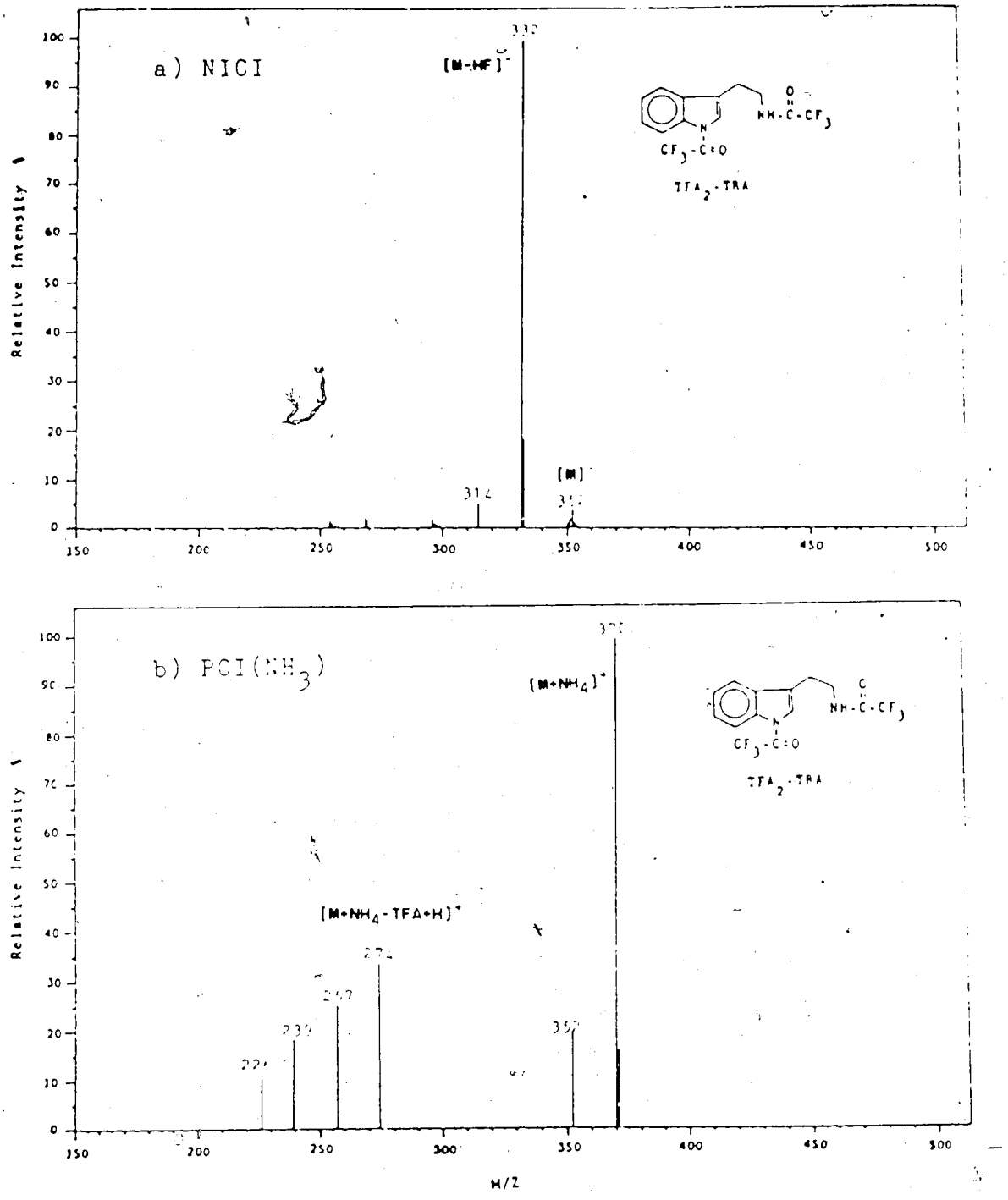


Figure 3.13 a). NICI spectrum of TFA derivative of TRA.
b). PCI(ammonia) spectrum of TFA derivative of TRA.

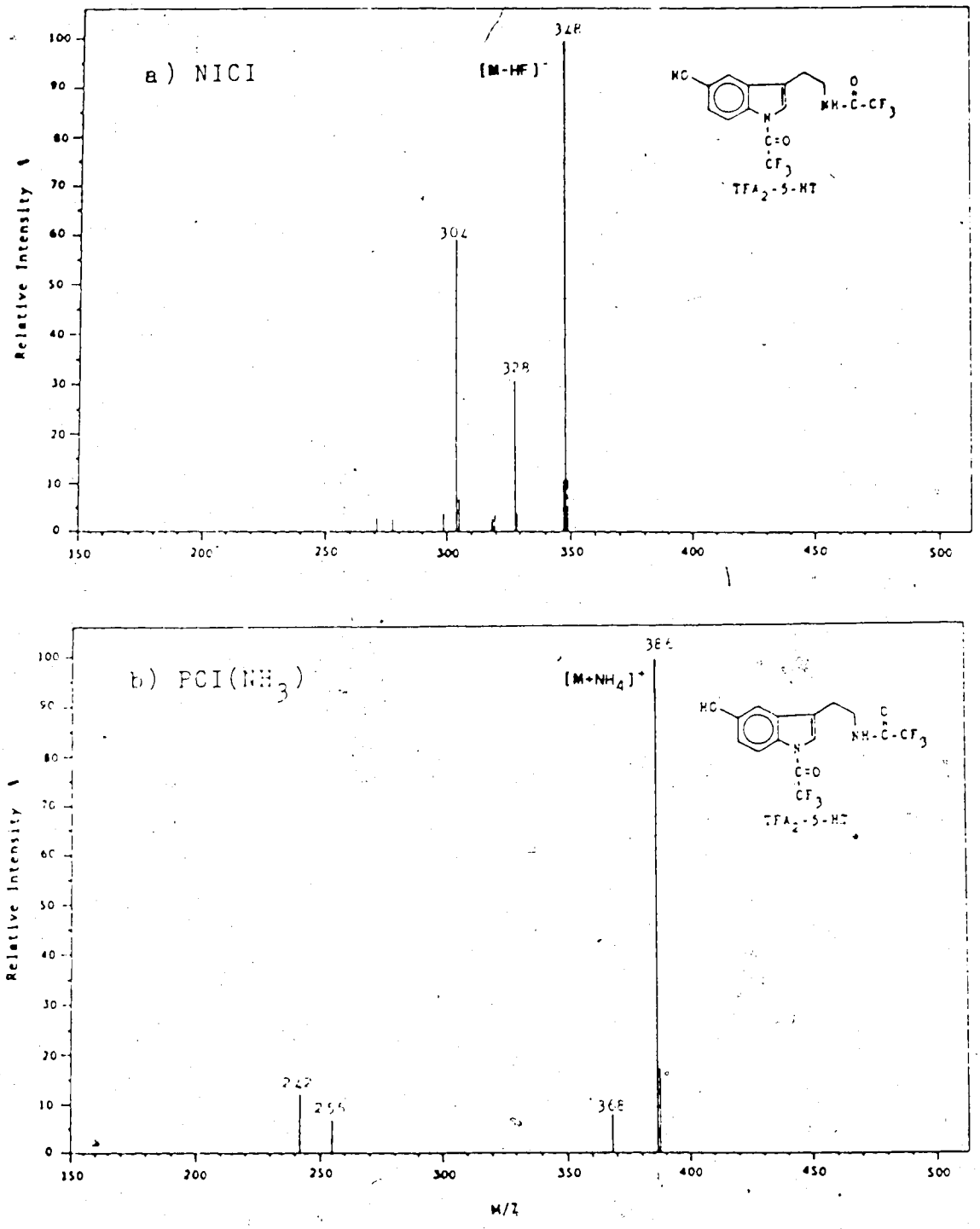


Figure 3.14 a). NICI spectrum of TFA derivative of 5-HT.
b). PCI(ammonia) spectrum of TFA derivative of 5-HT.

3.3. SENSITIVITY COMPARISION BETWEEN SEVERAL IONIZATION MODES IN GC-MS

To compare the sensitivity of the various ionization modes, the same amounts of the mixture of TFA derivatized 50 p mole of DA, P-OCT, M-OCT, SYNE, NMDA, SALSOL, TRA and 5-HT, and 0.5 n mole of DHPG were introduced into each individual ionization mode. The total ion chromatograms were recorded and shown through Fig. 3.15 to Fig. 3.18. It is clear from these figures that the sensitivity obtained with NICI is highest. The methane PCI gives strong background ions in TIC chromatogram, see Fig. 3.16. The situation is much worse in ammonia PCI where no peak can be identified. The order of the sensitivity is NICI > EI > PCI(methane) > PCI(ammonia). The high sensitivity with NICI results from the ability of the TFA derivatives of biogenic amines to capture near thermal electrons and form negative ions. At the same time no negative ions are produced from methane. Thus the NICI is very selective. On the other hand abundant positive ions are produced from CH_4 and NH_3 in the positive ion mode. The rate constant for formation of a negative ion by capture of a low energy electron can be as high as 400 times greater than that for an ion molecule reaction in PCI. Therefore, negative ion chemical ionization should facilitate detection and quantification of many organic molecules by two or three orders of magnitude compared with PCI or EI[27,28].

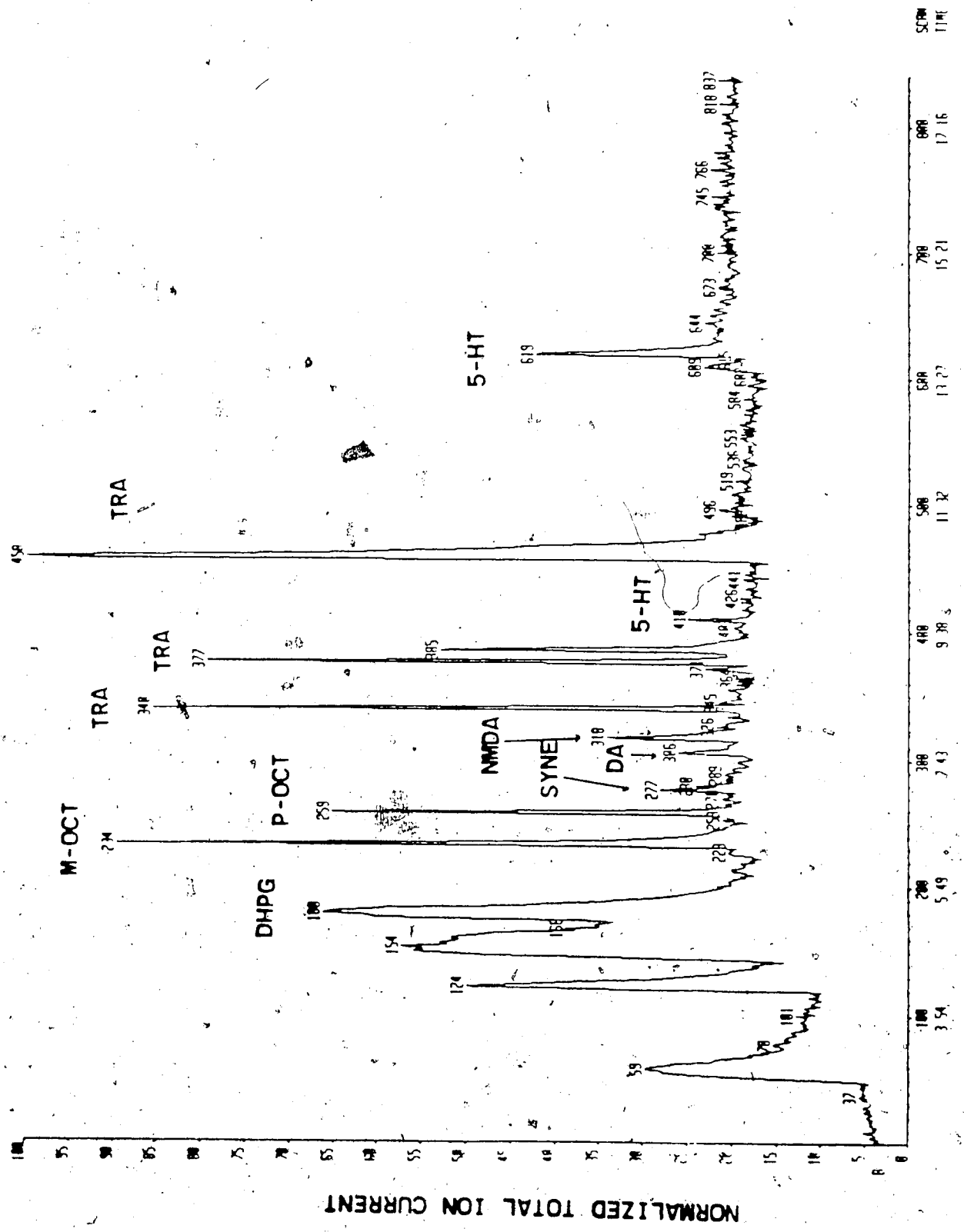


Figure 3.15 NICI TIC chromatogram of mixture of TFA derivatives of DHPG, M-OCT, P-OCT, SYNE, DA, NMDA, TRA, and 5-HT.

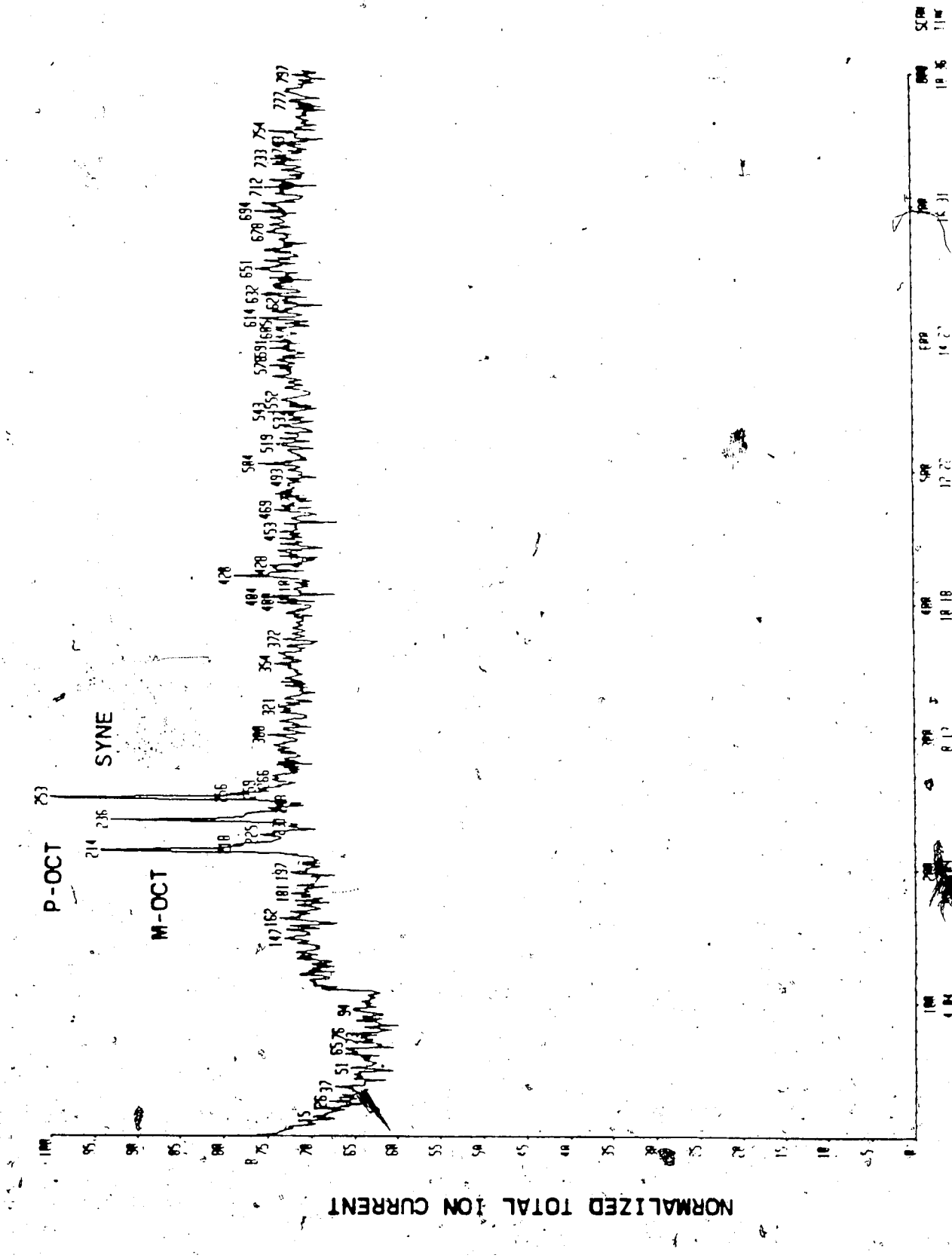


Figure 3.16 PCI(methane) TIC chromatogram of mixture of TFA derivatives of DHPG, M-OCT, P-OCT, SYNE, DA, NMDA, TRA, and 5-HT.

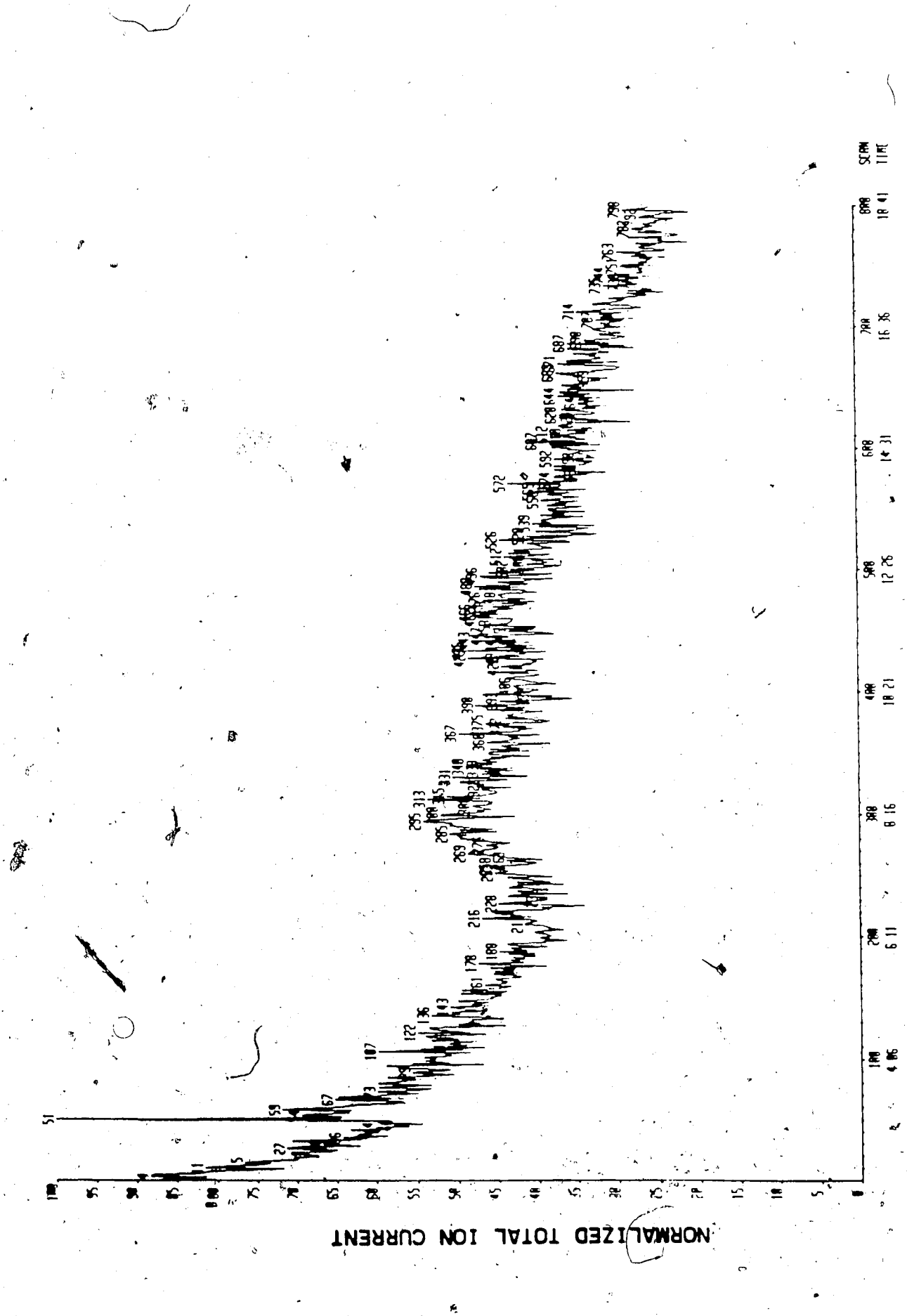


Figure 3.17 PCI(ammonia) TIC chromatogram of mixture of TFA derivatives of DHPG, M-OCT, P-OCT, SYNE, DA, NMDA, TRA, and 5-HT.

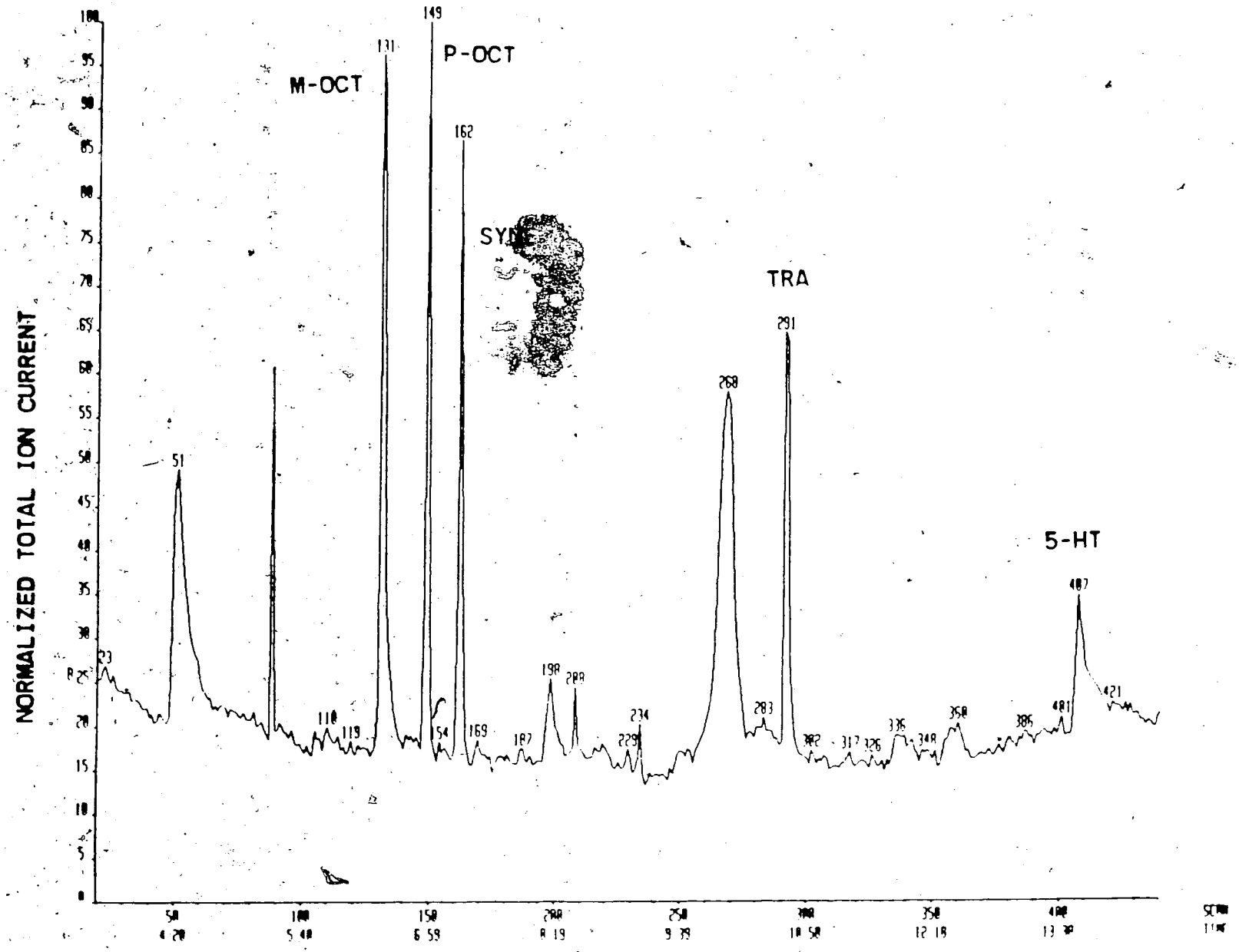


Figure 3.18 EI TIC chromatogram of mixture of TFA derivatives of DHPG, M-OCT, P-OCT, SYNE, DA, NMDA, TRA, and 5-HT.

CHAPTER 4

IDENTIFICATION AND QUANTIFICATION OF NEUROTRASMITTER CANDIDATES IN NERVE RICH TISSUES OF POLYORCHIS PENICILLATUS

The procedures including alumina extraction and HPLC-EC were done by my coworker, Jun-Mo Chung who is currently studying Neurophysiology in Department of Zoology, University of Alberta. Figure 4.1 and Figure 4.2, and Table 4.1 to Table 4.3 were kindly provided by him. He is responsible for the experiment and interpretation of these results.

4.1. PREPURIFICATION FROM THE TISSUE HOMOGENATE

Due to the complexity of the matrix of nerve rich tissues, it is necessary to apply prepurification techniques before introducing sample homogenates to HPLC. Among several preseparation methods, aluminum oxide is widely used for prepurification of catecholamines of tissue extracts[18,20,41]. The prepurification is based on adsorption of catecholamines onto alumina in alkaline medium. The alumina is then washed with water and the catecholamines desorbed by acidification. The extraction results from adsorption due to attraction between the alumina matrix and the two vicinal hydroxyl groups on the benzene ring of the catechol molecules[78]. The presence of

an amine group seems unimportant for the extraction. As a result, all catechols, not just catecholamines, are extracted by the alumina.

In the present study, this alumina extraction was applied to extract all catechol compounds from the tissue homogenate. Either a column or a batch technique can be used for the alumina extraction. In the present study, the batch technique was used because the entire assay can be done in one sample tube, and the final volume of each sample after extraction procedure was small enough so that it could be subjected to HPLC. However, in applying this technique to the nerve rich tissue, large variations in recoveries were observed. To correct for these variations, dihydroxybenzylamine (DHBA) was used as an internal standard.

The HPLC chromatograms of several catecholamines, metabolites and DHBA are shown before alumina extraction (Fig. 4.1 (a)) and after alumina extraction (Fig. 4.1 (b)). As expected, the compounds which do not have a catechol group in the molecule such as 5-HIAA, HVA and 5-HT (see Fig. 4.1 (a)), were not extracted by the alumina and are missing in chromatogram (b). Even though there is no amine in the molecular structure, a compound which has a catechol structure, such as DOPAC, can pass through this extraction step. The efficiency of the alumina extraction was evaluated by comparing the peak heights of alumina extracted and unextracted samples

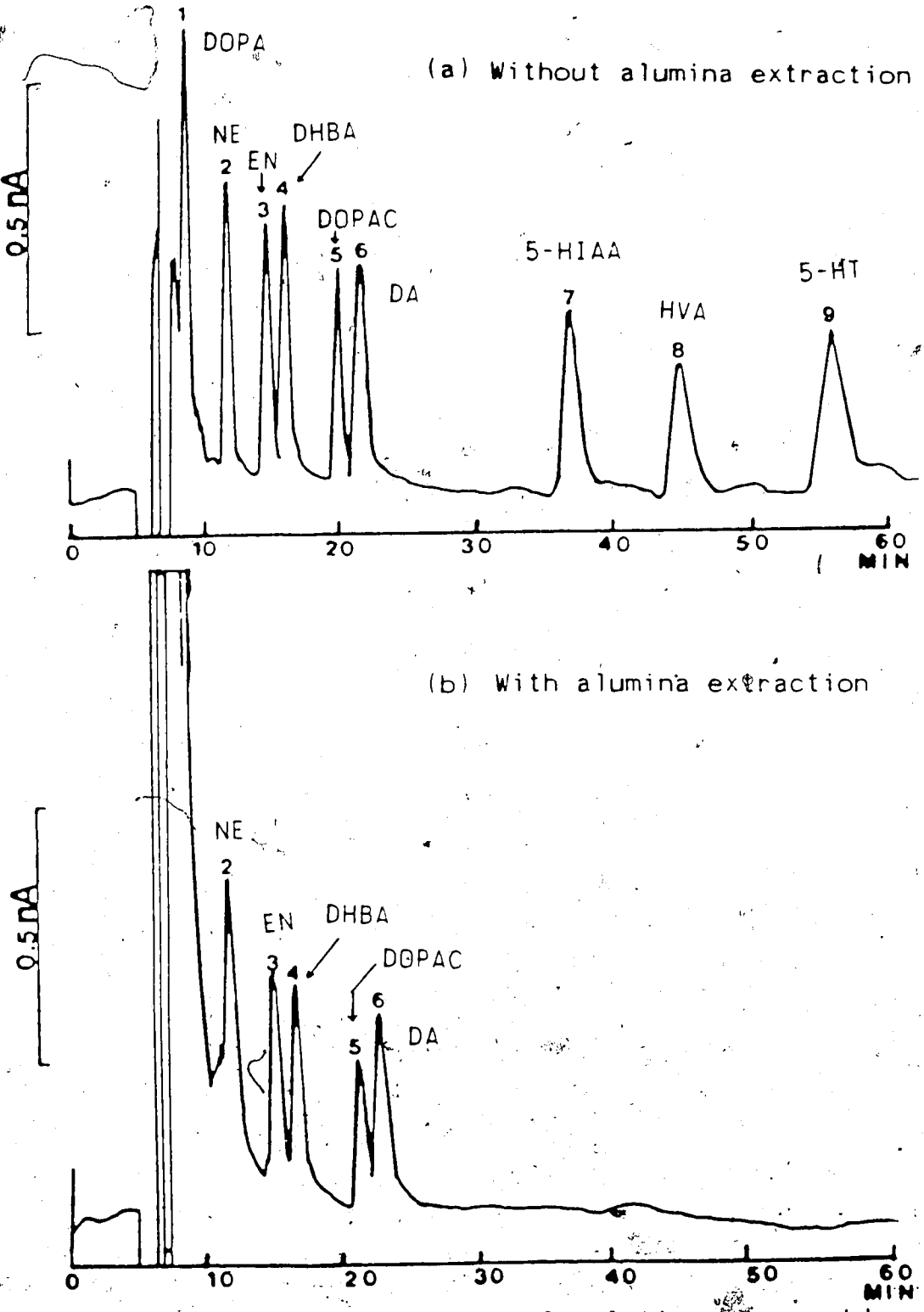


Figure 4.1 HPLC-EC Chromatograms of solution prepared by combining several authentic biogenic amines and metabolites without alumina extraction(a) and with alumina extraction (b). Compounds: 1:DOPA, 2:NE, 3:EN, 4:DHBA, 5:DOPAC, 6:DA, 7:5-HIAA, 8:HVA, 9:5-HT.

containing initially known amounts of catecholamines and DHBA. As shown in Table 4.1.A., the alumina extraction step recovered 65-80% of the compounds. The effect of tissue on the alumina extraction was also found by adding 5 pmole of catecholamines and DHBA to the samples with or without tissue, and by extracting catecholamines and DHBA through the alumina extraction step. The percent recovery yield is shown in Table 4.1 B.

4.2. IDENTIFICATION AND QUANTITATIVE DETERMINATION OF NEUROTRANSMITTER CANDIDATES BY HPLC-EC

The retention times of several catecholamines and DHBA after alumina extraction with the tissue matrix and without tissue matrix in HPLC-EC are listed in Table 4.2. The typical chromatogram of a nerve rich tissue extract prepared by alumina extraction is shown in Fig. 4.2. The front big peak in Fig. 4.2 is due to the 1.0 M hydrochloric acid and sodium metabisulfate. This peak was sometimes big enough to interfere the retention region of DOPA. When 0.1 M HCl acid was used to desorb the catechol compounds from the alumina, the big front peak was reduced in intensity in the EC detector. But 0.1 M HCl was not used for the entire experiments because it caused poor recovery of catechol compounds from the alumina extraction. The EC detector does not respond to the TRIS at a potential of 0.75V vs. a Ag/AgCl reference electrode. However, a large amount of TRIS passed through the alumina extraction. Introducing the

TABLE 4.1

Recoveries of the Catecholamines as a Function of Alumina Extraction and of Presence of Tissue[a].

	NE	EN	DA	DHBA
A. Efficiency of alumina extraction				
Extracted sample	0.69±0.02	0.62±0.04	0.51±0.05	0.74±0.04
Unextracted sample	1.04±0.02	0.88±0.02	0.71±0.01	0.94±0.02
Percent Recovery	66.4±0.6	70.5±2.9	71.8±6.5	78.7±2.5
Percent Recovery relative to DHBA	84.3±1.9	89.5±0.8	91.3±5.1	100
B. Effect of tissue on Recovery[b]				
Percent Recovery (N=5)	90.1±7.8	98.8±2.0	92.4±0.9	98.7±5.1

[a] Values given are in mean peak response(nA) ± SD, N=10 in all cases except the case of effect of tissue on recovery experiment.

[b] The extent of recovery was found by adding 5 pmole of catecholamines and DHBA to the sample without or with tissue from 1 to 3 animals.

TABLE 4.2

Retention Times of the Catecholamines and DHBA ON HPLC-EC in Chromatography System I[a]

	NE	EN	DHBA	DA
A. WITHOUT TISSUE				
Unextracted Standard	12.13±0.20	15.45±0.21	17.02±0.17	23.38±0.20
Extracted Standard	12.13±0.09	15.38±0.11	16.92±0.15	23.32±0.08
B. WITH TISSUE				
Extracted Standard (N=5)	12.25±0.25	15.33±0.22	16.89±0.13	23.40±0.21

[a] Values are expressed in minutes (mean ± S.D.) and obtained in system I
N=10 in all cases except in the case of with tissue experiment

System I: The compounts of mobile phase are 55mM sodium phosphate(monobasic), 0.85mM sodium octyl sulfate, 7mM disodium EDTA and 9% acetonitrile. The pH of the mobile phase was 3.75. Econosphere RP-18 Column was used.

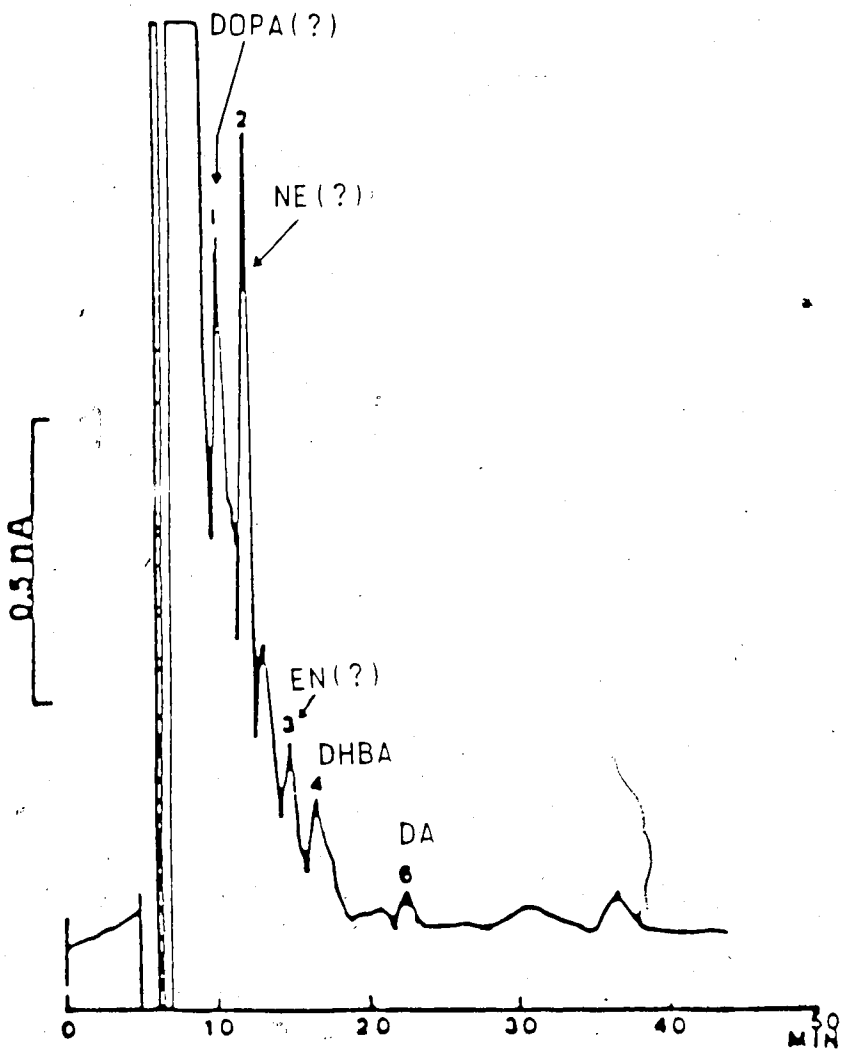


Figure 4.2 HPLC-EC Chromatogram of a real tissue extract sample in chromatography system I. (System I: see the experimental section)
Compounds: 1:DOPA like compound, 2:NE like compound, 3:EN like compound, 4:DHBA, 6:DA.

alumina extract without using antioxidant such as sodium metabisulfate into the HPLC-UV, it was found that there was still a big front peak but this was not due to the HCl. By introducing TRIS into HPLC-UV, it was found that TRIS has good response in the UV detector and the retention time of TRIS is matched with the big peak in the UV chromatogram of the alumina extract.

By comparing the retention times of several peaks in a nerve rich tissue chromatogram with those when authentic compounds were added to the tissue matrix, it was found that dopamine(DA) and several other compounds(NE, EN, and DOPA) had similar retention times. In Fig. 4.2, the peak which is assigned as #6, seems to be DA, because its retention time is the same as that of authentic dopamine. The concentration of dopamine in the nerve rich tissue was calculated by the following formula and the result is listed in Table 4.3.

$$\text{Conc. of CA} = \frac{\text{P.H. ratio of CA/DHBA for sample}}{\text{P.H. ratio of CA/DHBA for stand.}} \times \frac{[IS]}{SA}$$

CA : Catecholamine

P.H.: Peak Height

[IS] : The concentration of internal standard solution.

SA : The ratio of the sample volume to the volume of the internal standard solution.

TABLE 4.3

Identification and Quantification of Catecholamines in the nerve rich tissue of Polyorchis penicillatus.

NE	EN	DA
NI/NQ[a]	NI/NQ	0.12 ± 0.06[b]

[a] NI: not idetified NQ: not quantifiable

[b] Values are expressed in p mole ± S.D. per mg of wet tissue weight.

The peaks assigned #1, #2 and #3 in Fig. 4.2 seem to come from DOPA, NE and EN, respectively. Even though their retention times are very similar to those of the authentic compounds, one can not be certain that these peaks are due to DOPA, NE and EN. By introducing crude nerve rich tissue homogenate into the HPLC-UV system (Chromatographic system II), it was observed that a large number of compounds elute from the column with strong intensities in the catecholamine region. Even though they are not selectively adsorbed to the alumina like the catechol compounds, their higher concentrations seem to be high enough to make them pass through the alumina extraction step and interfere in the regions where real catecholamines elute. Therefore, the presence of the catecholamines can not be proven on the basis of retention times. But DA elutes away from the other catecholamines so the presence of DA can be proven by its retention time. More selective experiments such as a mass spectrometric detection is needed to study the front region of the chromatogram and to confirm the presence of dopamine.

4.3. IDENTIFICATION OF NEUROTRANSMITTER CANDIDATES BY GAS CHROMATOGRAPHY NEGATIVE ION CHEMICAL IONIZATION MASS SPECTROMETRY

Several authentic catecholamines and DHBA were derivatized with TFAA in ethyl acetate and introduced into GC-MS(VG 7070E) in negative ion chemical ionization mode where methane was used

as the reagent gas. The resultant total ion current chromatogram is shown in Fig. 4.3. The retention times and major ion intensities of the NICI GC-MS separation of TFA derivatives of several catecholamines and DHBA are listed in Table 4.4. The GC temperature was 100 ^{o}C initially and was increased to 290 ^{o}C by ten degrees per minute. The compounds eluted at temperatures up to 240 ^{o}C. The increase from 240 to 290 ^{o}C was used in order to clean the column by baking it at a high temperature such as 290 ^{o}C.

Two kinds of GC retention times are listed. Table 4.4 (A) shows the retention times of several authentic compounds without sample matrix. The retention times of several authentic compounds with sample matrix are shown in Table 4.4 (B). The intensities of the molecular anion and the base peak are listed in the table. These two ions are used to obtain the selected ion current chromatograms. The ratio of these two ions from a real biological sample should be identical with the corresponding ratio from the authentic standard. But as we can see in the table, the ratio of these two peaks is slightly different.

It is interesting that the derivatized DHBA, the internal standard, also shows a good NICI response. The molecular weight of TFA derivatized DHBA is 427. The NICI spectrum of derivatized DHBA is shown in Fig.4.4. In the spectrum, there is an ion at 427 which is a molecular anion and there is an ion at

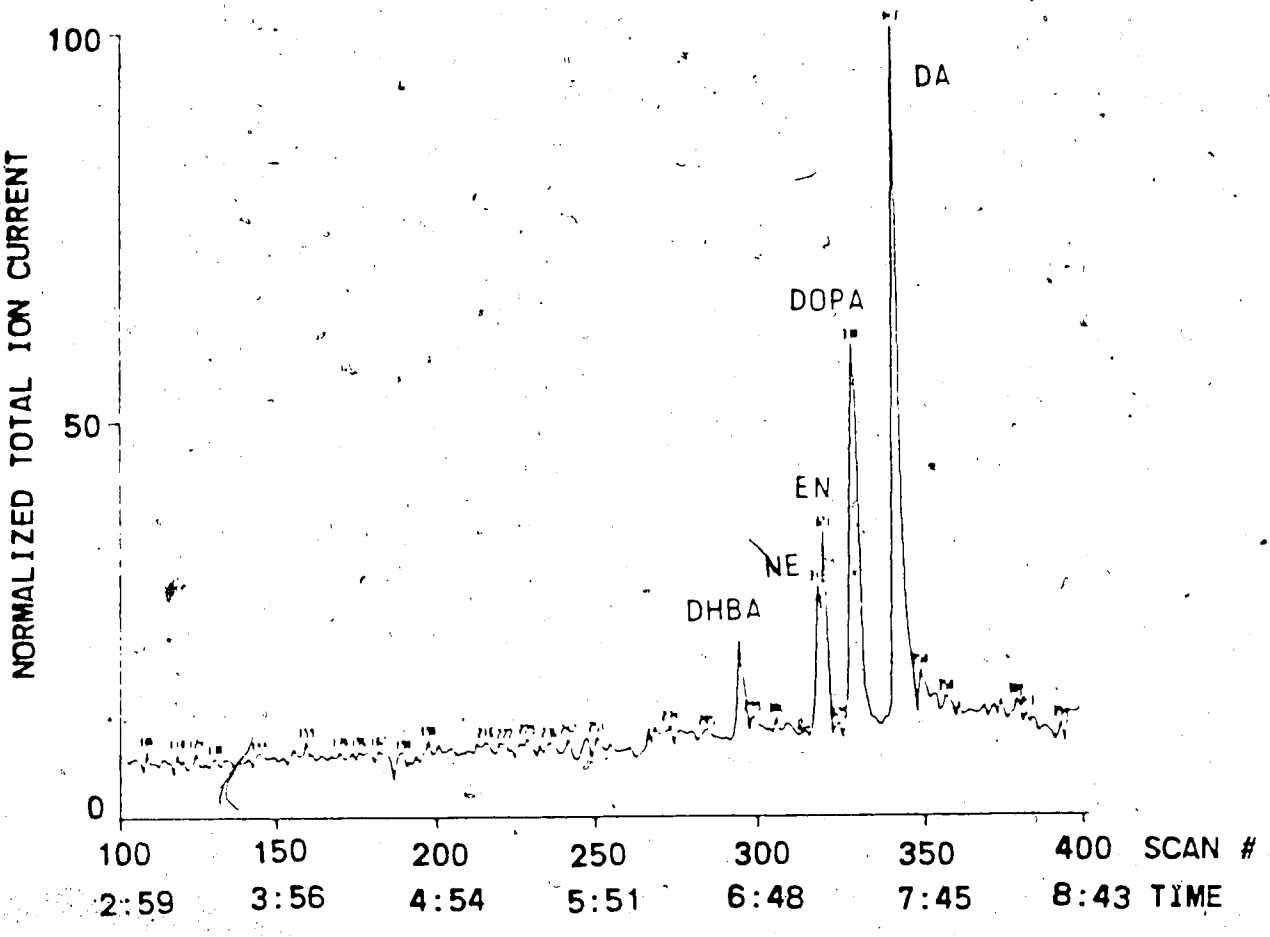


Figure 4.3 NICI GC-MS total ion current chromatogram of TFA derivatives of mixture prepared by combining several authentic catecholamines and DHBA.

TABLE 4.4

The GC-MS NICI Characteristics of the TFA derivative of several Catecholamines and DHBA[a]

TFA DERIVATIVE	M.W.	GC R.T. (MIN:SEC)	RELATIVE INTENSITY(%) M^-	$[M-97]^-$	RATIO OF $M^-/[M-97]^-$
A. WITHOUT TISSUE AND ALUMINA EXTRACTION					
DA	441	7:32	7.5	100	0.075
NE	553	7:05	10.7	67.4	0.159
EN	567	7:07	1.6	100	0.016
DOPA	467	7:17	8.5	100	0.085
DHBA	427	6:37	3.8	100	0.038
B. WITH TISSUE AND ALUMINA EXTRACTION[b]					
DA	441	7:50	4.9	100	0.049
DHBA	427	6:51	4.2	100	0.042

[a] GC-MS conditions: The temperature of GC oven was programmed to keep 100 °C initially and increased to 290 °C by 10 degrees per minute. SPB-5 GC column was used. The temperatures of GC injector was 240°C and ion source was 190 °C.

[b] 3 ul of sample was injected to GC injector.

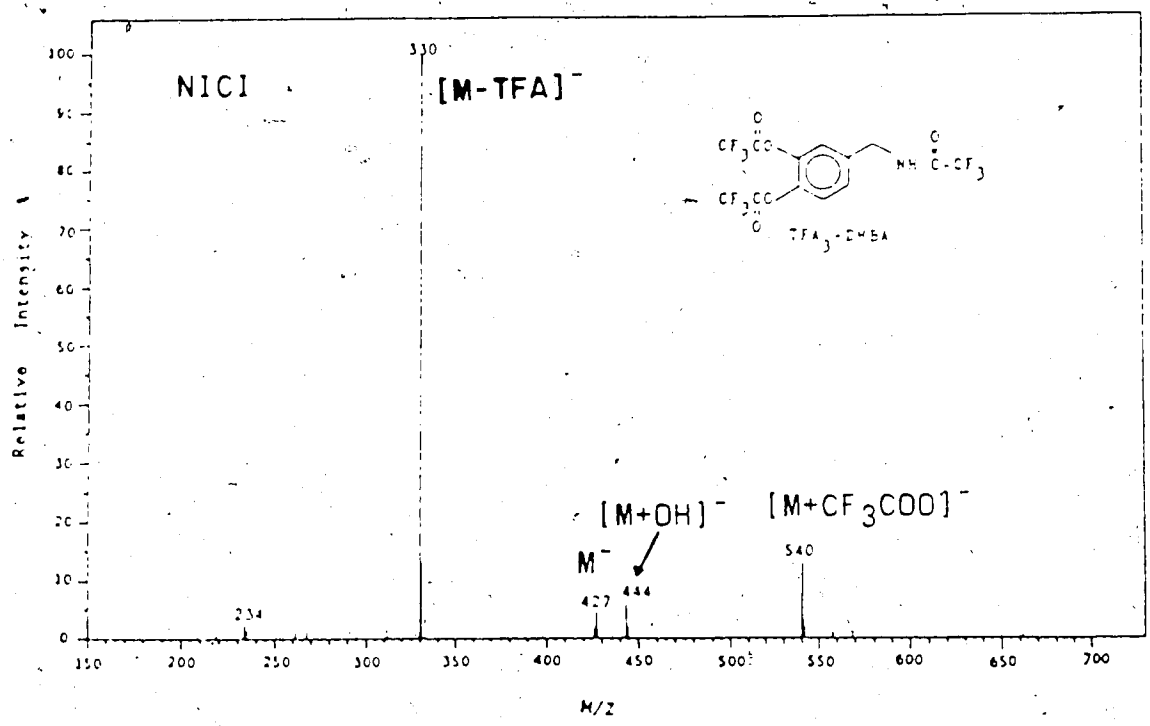
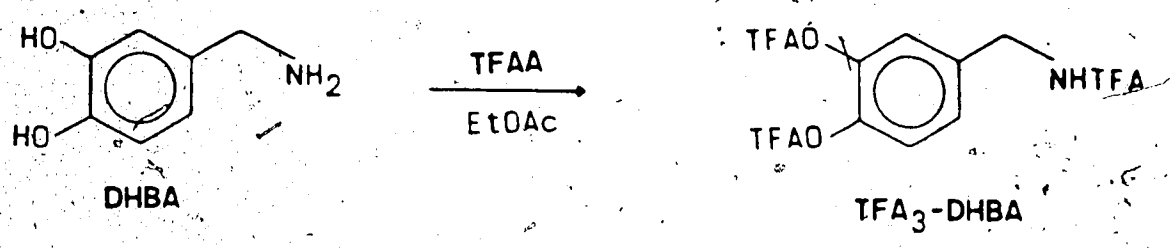


Figure 4.4 NICI spectrum of TFA derivative of DHBA (MW=427).

444 which is the OH^- cluster ion with the molecular anion.



The base peak of derivatized DHBA comes from elimination of a TFA group from the molecular anion as expected for catecholic compound derivatives (see Table 3.2).

Total ion current chromatogram of a real tissue extract sample is shown in Fig. 4.5. 1 ul of derivatized alumina extract was injected into the GC and the splitless mode was used. Before the tissue was homogenized, DHBA was added to the dissected nerve rich tissue of the jelly fish, to serve as internal standard in the HPLC-EC. Aluminum oxide was used to extract catechol compounds by adjusting pH of the homogenate alkaline with TRIS buffer. Because highly concentrated TRIS was used, there is a lot of TRIS left over in the alumina extract. TRIS is not detected in HPLC-EC. But the TFAA derivatized TRIS and then the products were detected in NICI-GC-MS. The huge tailing peaks up to scan number 120 which are in the front of the TIC chromatogram come from the TRIS trifluoroacetic derivatives. The NICI chromatogram of the TFA derivatives of an authentic TRIS is shown in Fig. 4.6 and their mass spectra are shown in Fig. 4.7. TRIS gives two major

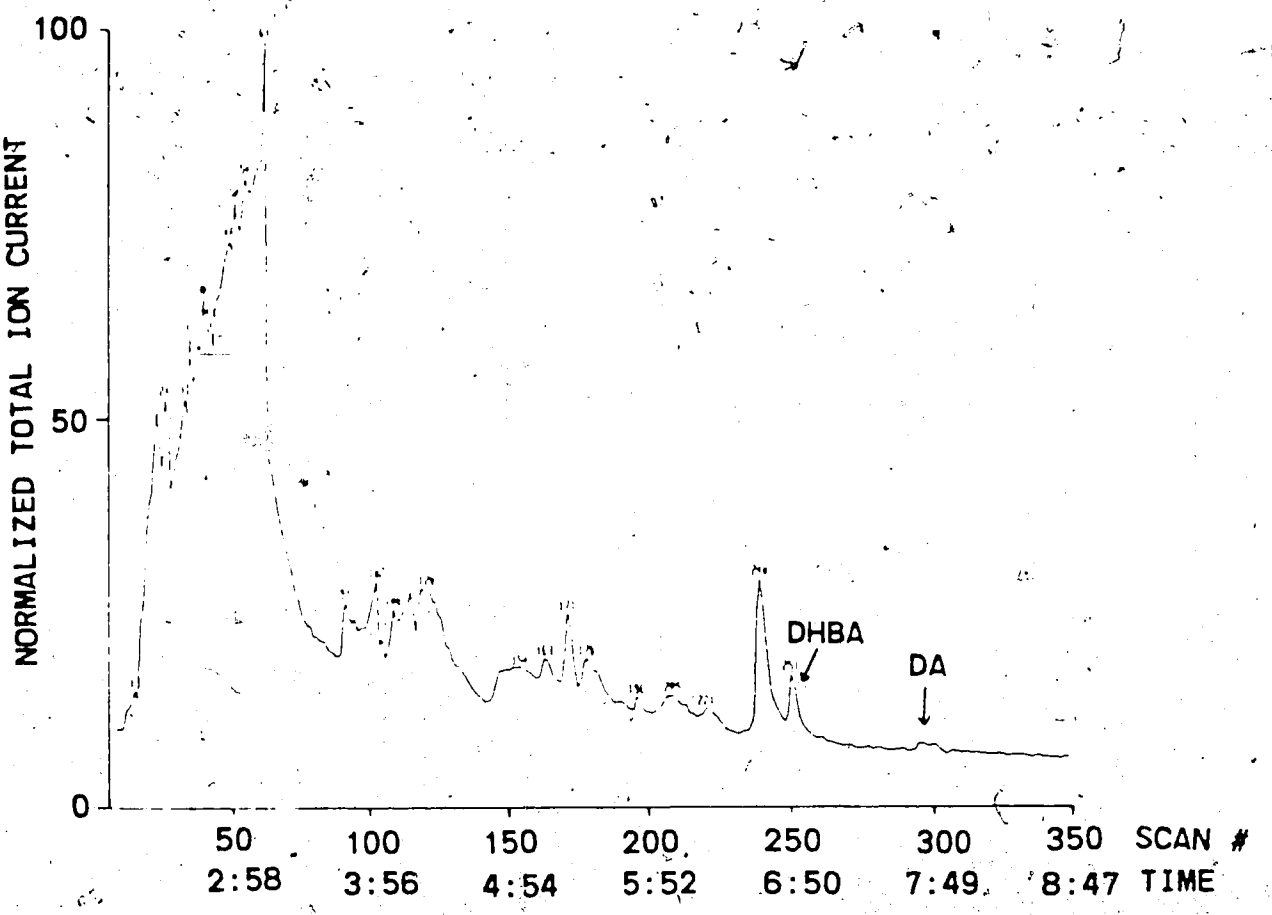


Figure 4.5 GC-MS NICI total ion current chromatogram of a real tissue extract sample.

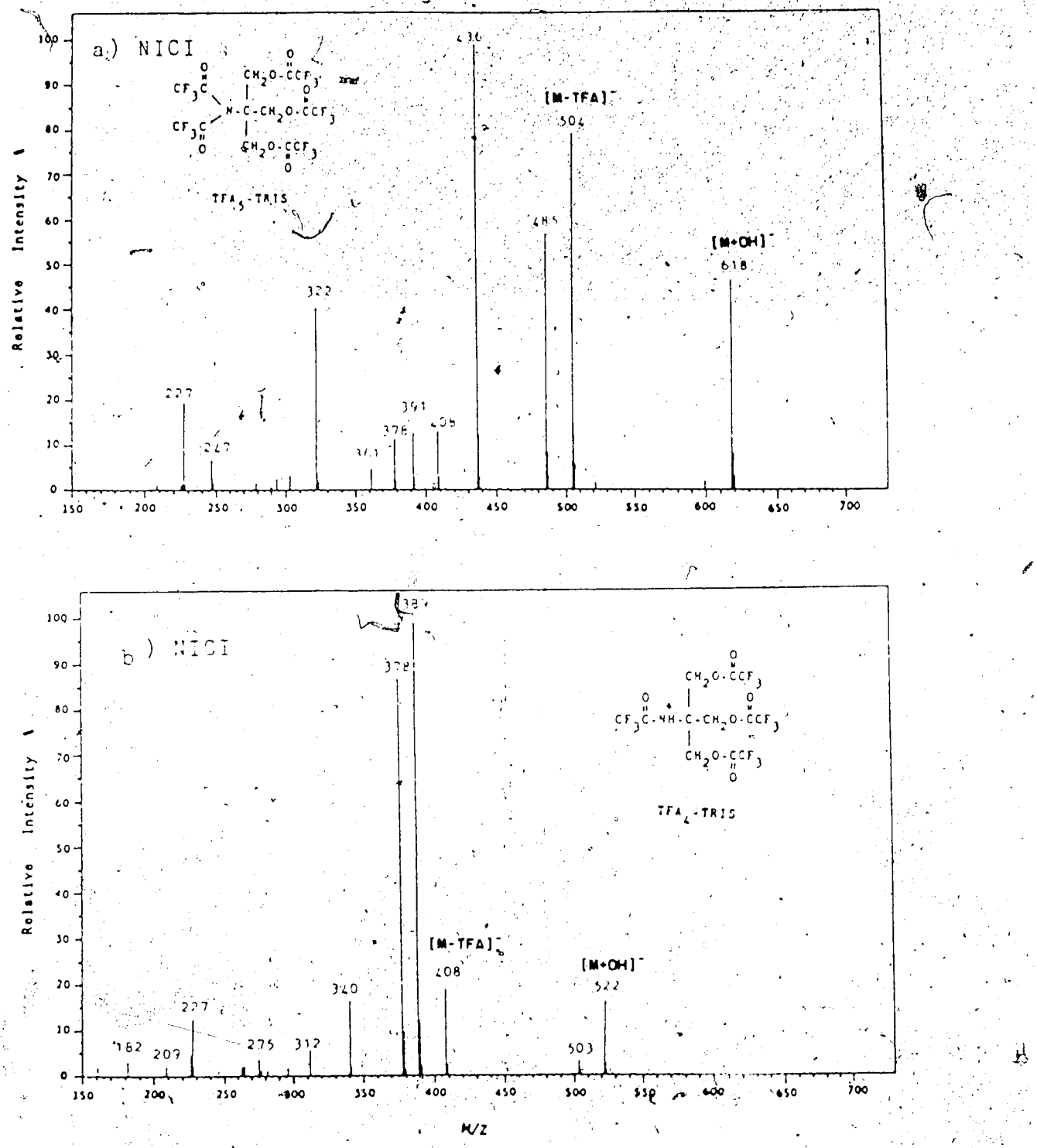


Figure 4.6 NICI spectra of two major TFA derivative products of TRIS. a) TFA_5-TRIS b) TFA_4-TRIS

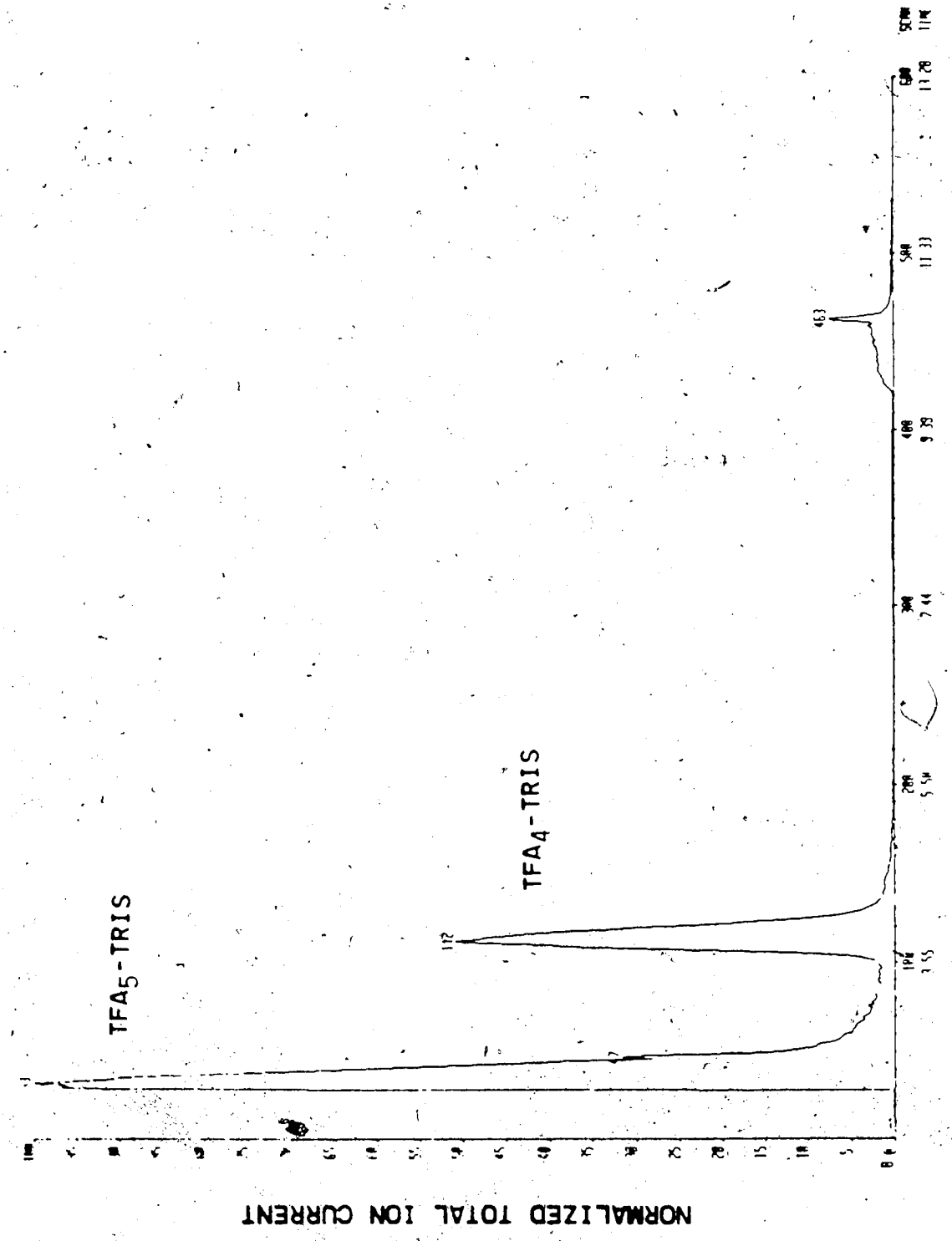
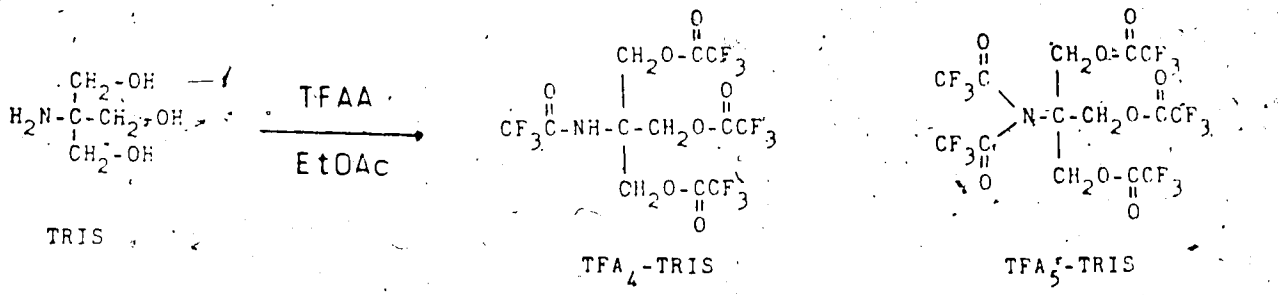


Figure 4.7 GC-MS NICI TIC chromatogram of the TFA derivative of TRIS.

products when it reacts with TFAA. One has molecular weight 601 and the other 505.



As seen from the spectra, there are no molecular anions. Instead, there are ions at 618 and 522 which correspond to the OH^- adducts to the respective molecules. Even though the mass spectra of TRIS in the TIC of a nerve rich tissue were saturated, there was no confusion to identify them because the major ions in the spectra of TFA derivatives of the authentic TRIS were present in those of the nerve rich tissue.

To check for the presence of DHBA derivative in the GC-MS spectrum of a nerve rich tissue, reconstructed selected ion chromatograms were obtained for the two informative ions of TFA derivative DHBA, from the TIC chromatograms. These are the ions at m/e=427 which is the molecular anion, and 330, the base peak. As shown in Fig. 4.8, it is clear that the peak, scan number 251, comes from DHBA derivative which was added as the internal standard.

To check whether there is dopamine in the tissue or not, selected ion chromatograms were reconstructed from the TIC of a

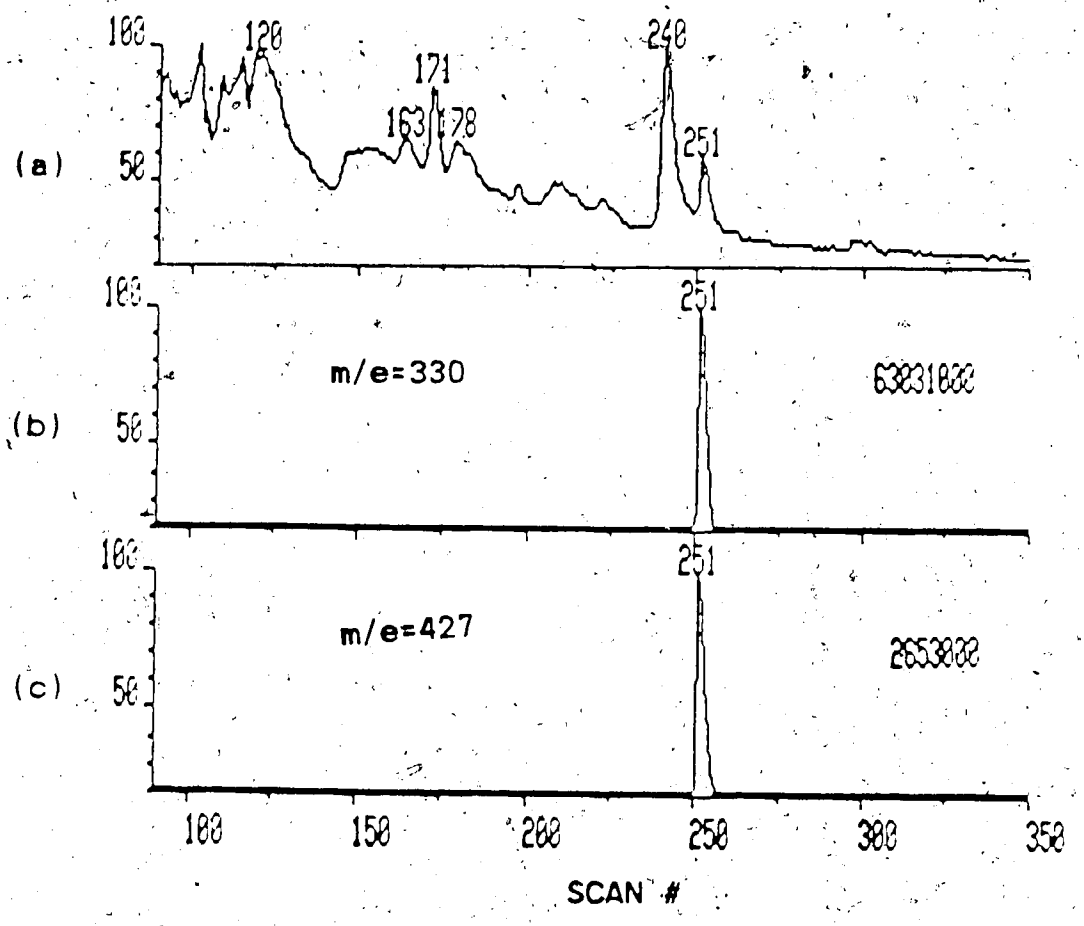


Figure 4.8 a). GC-MS NICI TIC chromatogram of a real tissue sample in scan #90-350.

b), c). Reconstructed Selected Ion Current chromatograms for ions at m/e=330 and 427.

nerve rich tissue sample(Fig. 4.9). Two ions, m/e=441 and 344, were selected. The ion at m/e=441 is the molecular anion of the TFA derivatized dopamine and the ion at m/e=344 is the base peak of dopamine NICI spectrum (Fig. 3.1). From these two selected ion chromatograms, it can be easily shown that the peak at scan number 297 corresponds to the TFA derivatized dopamine. The intensity ratio of the two ions (441/344) in the chromatograms is 0.043 but the ratio of the authentic compound is 0.049 (Table 4.4). This variation is probably due to the inconsistency of the ion source pressure. There is a little difference in GC retention times of dopamine between DA in a nerve rich tissue extract and authentic DA in the tissue matrix. The retention time of DA in a nerve rich tissue extract is 7 minutes and 55 seconds. The retention time of authentic dopamine is 7 minutes and 50 seconds when the tissue sample was spiked with authentic dopamine. The NICI spectrum of scan number 297 is shown in Fig. 4.10. This spectrum can be compared with the spectrum of authentic dopamine(Fig. 3.1 (a)). In the spectrum, there is not an ion at m/e=458 which corresponds to $[M+17]^-$ but when 3 ul instead of 1 ul solution was introduced into the GC injector, an ion at m/e=458 whose intensity was twice as high as that of the molecular anion was observed. By comparing the spectrum of scan number 297 of TIC chromatograms of the nerve rich tissue extract (Fig. 4.10) with that of authentic dopamine standard(Fig. 3.1 (a)), the peak at scan number 297 is certainly due to the TFA derivatized dopamine in the nerve rich tissue. Even though there are several unmatching peaks in the nerve rich tissue

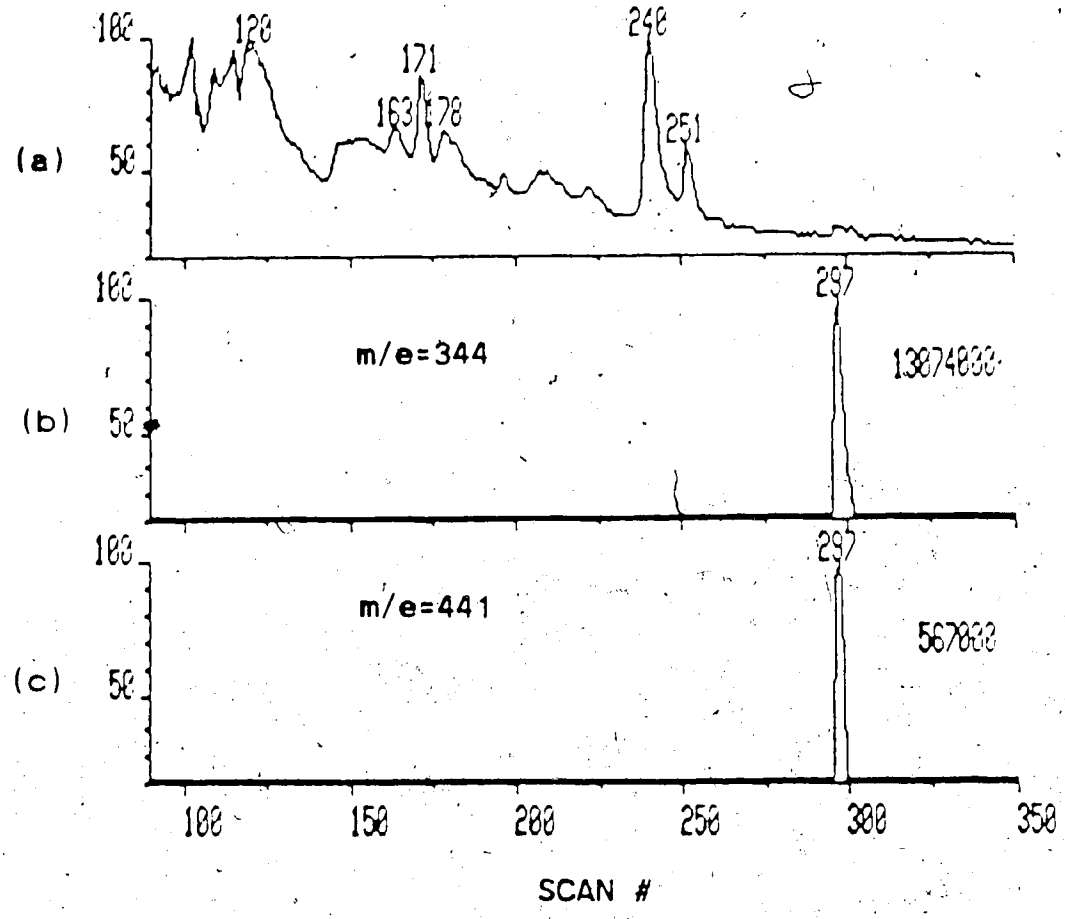


Figure 4.9 a). GC-MS NICI TIC chromatogram of a real tissue sample in scan #90-350.

b), c). Reconstructed Selected Ion Current chromatograms for ions at m/e=344 and 441.

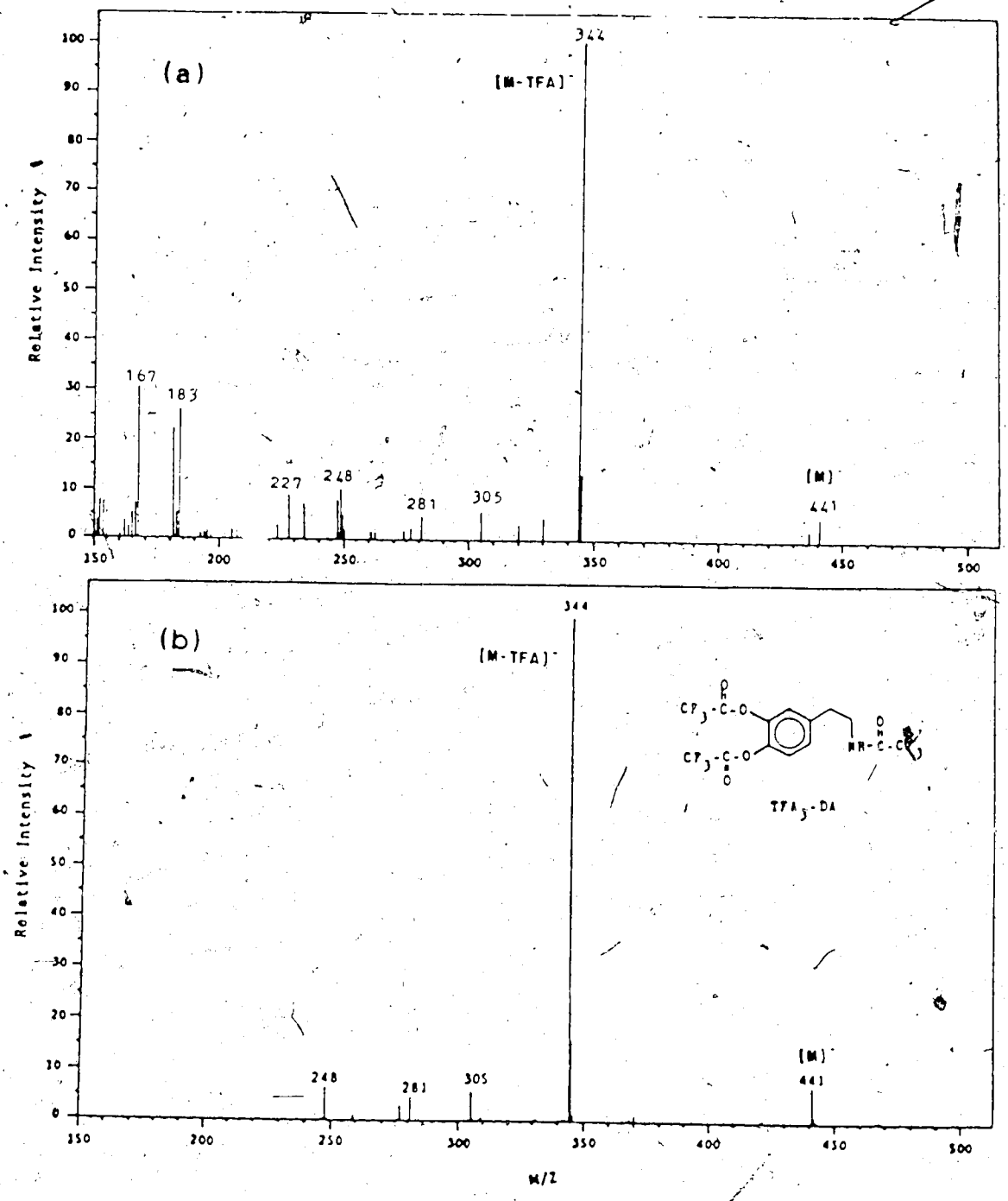


Figure 4.10 (a) $NICI_3$ spectrum of scan number 297 in the TIC chromatogram of a real tissue extract sample. (b) NICI spectrum of TFA derivative of authentic DA.

extract, major ion peaks at m/e=441, 344, 305, 281, and 248 in the authentic standard are found also in the spectrum of the tissue sample. The peaks at m/e=227, 183, 181, and 167 in Fig. 4.10, that do not match, could be due to strong background ions, because these peaks were observed also in the spectra at other scan numbers. Three different tissue samples were used and the spectrum in Fig. 4.10 was reproducibly observed.

For confirmation of the existence of NE in the nerve rich tissue extract, reconstructed selected ion current chromatograms were obtained for ions of m/e= 456 $[M\text{-}TFA]^-$, 439 $[M\text{-}TFAOH]^-$, and 553 $[M]^-$ from the total ion current chromatogram (Fig. 4.11). From these chromatograms, it is evident that NE is not present. Even though Fig. 4.11 (c) shows that there is a little possibility of existence of NE, the mass spectrum of scan number 268 shows that the ion at m/e=456 is in the noise level of the entire spectrum. Fig 4.11 (d) shows that it is evident that there is no NE in the nerve rich tissue sample above the level of detection limit of GC-MS which is approximately 1 p mole per injection.

Several reconstructed chromatograms were obtained for characteristic ions of m/e=567 $[M]^-$, 470 $[M\text{-}TFA]^-$, and 356 $[M\text{-}TFA\text{-}TFAOH]^-$ from the TIC chromatogram of a nerve tissue sample (Fig. 4.11). The NIC spectrum of the TFA derivatized authentic EN is shown in Fig. 3.7 (b). As seen in the figure, there is no EN in the nerve rich tissue extract. In HPLC

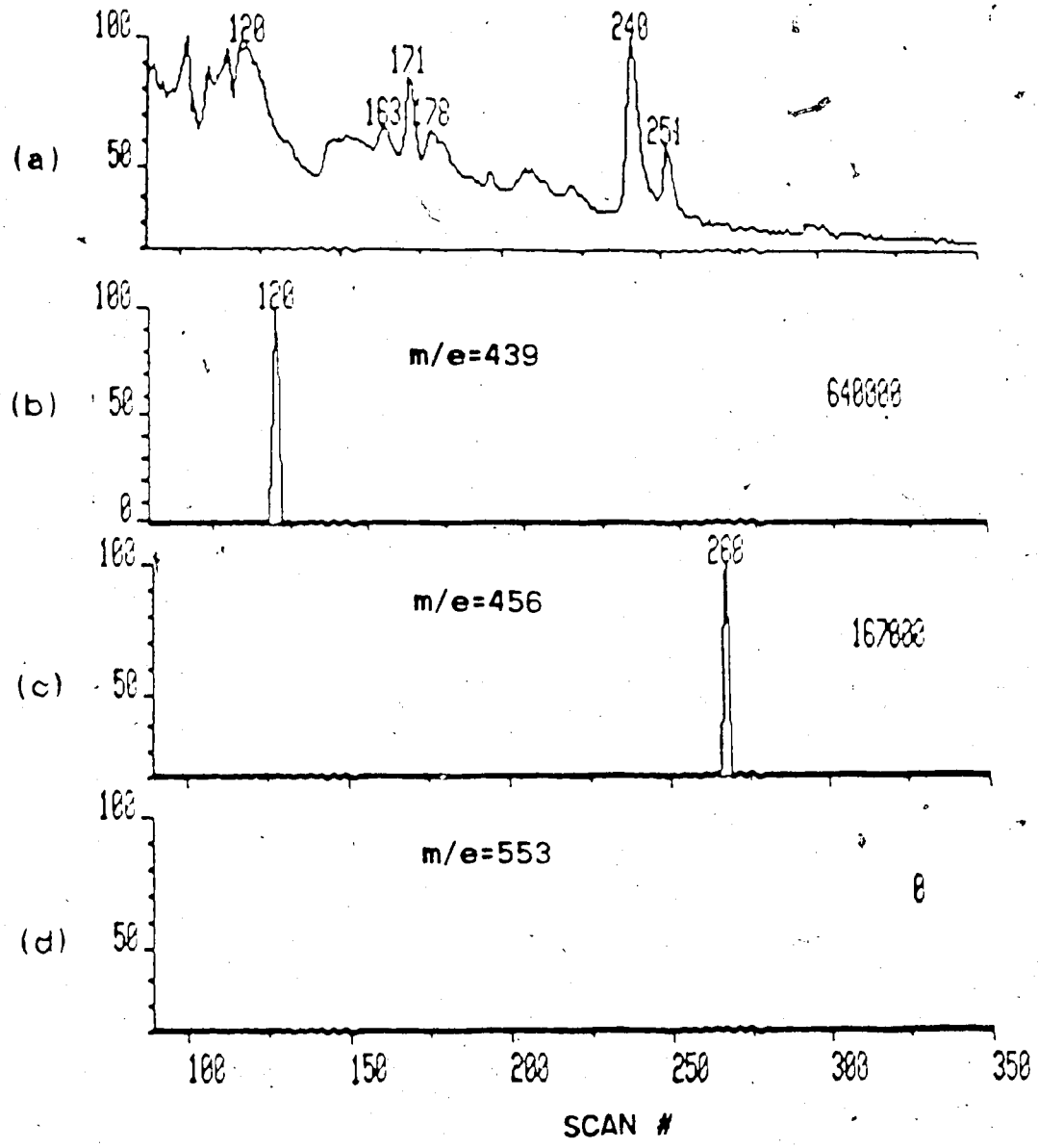


Figure 4.11 a). GC-MS NICI TIC chromatogram of a real tissue sample in scan #90-350.

b), c), d). Reconstructed Selected Ion Current chromatograms for ions at m/e=439, 456, and 553.

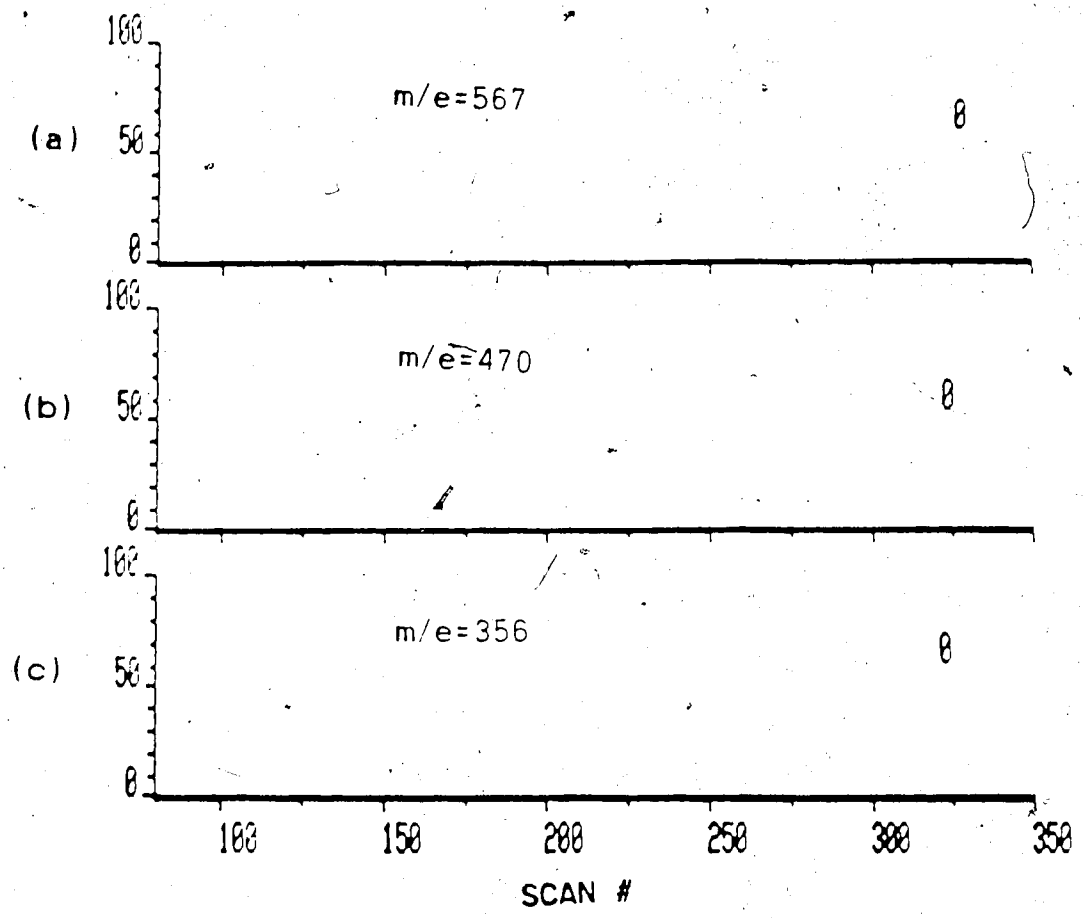


Figure 4.12 a),b),c). Reconstructed Selected Ion Current chromatograms for ions at m/e=567, 470 and 356.

chromatograms of the tissue extract, peaks with retention times equal to those for NE and EN were observed, see Fig.4.2. In the light of the present results, these peaks i.e. #2 and #3 in the HPLC chromatogram (Fig. 4.2) should be regarded as unknowns.

DOPA is the immediate precusor of DA in the conversion from tyrosine (Fig. 1.1). Because DA is found to exist in the nerve rich tissue, DOPA should also be expected to exist in the nerve tissue. But in the GC-MS experiment, no DOPA was found. The NICI spectrum of the authentic standard DOPA derivative is shown in Fig. 3.9 (a). For the two major ions, the molecular anion at m/e=467, and the base peak ion at m/e=370 $[M-97]^-$, reconstructed selected ion current chromatograms were obtained from the TIC chromatogram (Fig. 4.13). As seen in those figures, the currents for the two ions were zero intensity. This result shows quite conclusively that DOPA was not present in the tissue sample at least above the detection limit i.e. approximately 1 p mole per injection. This corresponds to less than 200 picogram DOPA per 0.1 gm wet weight of nerve rich tissue.

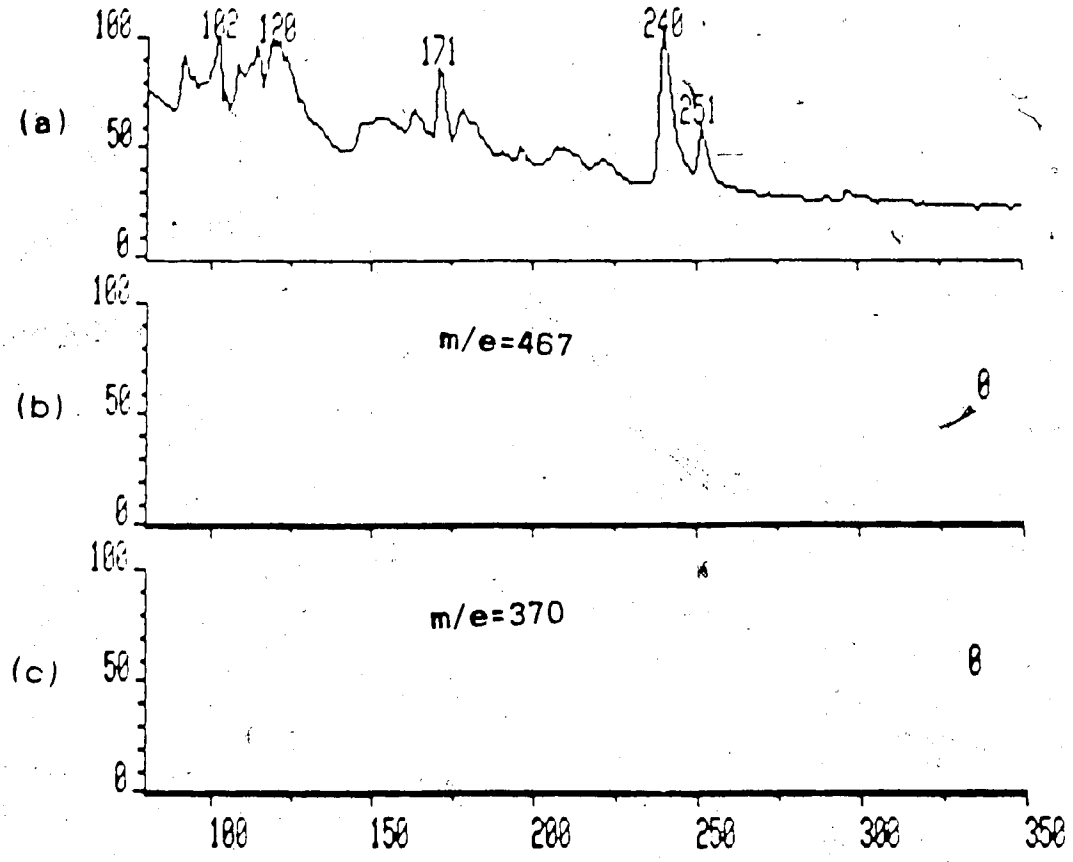


Figure 4.13 a). GC-MS NICI TIC chromatogram of a real tissue sample in scan #90-350.

b),c). Reconstructed Selected Ion Current chromatograms for ions at m/e=467 and 370.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FURTHER EXPERIMENTS

5.1. CONCLUSIONS

Mass spectra of several biogenic amines, metabolites, and several related compounds were obtained in the several ionization modes. Each ionization mode has its own merits and defects. Even though the sensitivity of ammonia PCI was the lowest among all ionization modes investigated, this ionization clearly provided information for the molecular weight determination of the analytes. The methane PCI, which is more sensitive than ammonia PCI, gives supplementary information about the molecular weight and the molecular structure. Although EI is less sensitive than NICI, it gives abundant peaks in the spectra of analytes, providing in this manner information about the structure of the analytes. NICI is the most sensitive mode among all ionization modes, although it provides little structural information. Combined with capillary column gas chromatography, NICI provided the necessary tool in the search for biogenic amines present at picomole levels in nerve tissue.

As a practical application an assay for catecholamines in a nerve rich tissue of Polyorchis penicillatus was undertaken.

By combining GC-MS with HPLC-EC picomole levels of dopamine were found to exist in mg. wet weight of a nerve rich tissue of Polyorchis penicillatus. The finding of dopamine in the nerve rich tissue of coelenterate is of special biological significance. The existence of dopamine strongly suggests that there are chemical synapses in the nerve systems of coelenterate aminals.

5.2. SUGGESTIONS FOR FURTHER EXPERIMENTS.

Even though dopamine was found in the nerve rich tissue of Polyorchis penicillatus, several more experiments should be done to confirm that dopamine is a neurotransmitter and to find out the precusor and metabolites of dopamine. In this present experimental procedure, it is impossible to detect metabolites of dopamine that do not have a catechol group in the molecular structures. Therefore, a different procedure, not using alumina extraction, is needed to study the metabolites and precusor. The direct coupling of HPLC with MS will be useful in this kind of experiment. By using HPLC-MS, it will be also easy to study the frontal part of the HPLC chromatogram where there are a lot of unknown peaks. The proper mobile phase should be chosen to sussessful coupling of HPLC-MS. Experiments of this type are now in the exploratory stage in this laboratory.

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