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Development of HPLC-EC Methodology & Its Use in the Determination of the Daily Pattern of Brain Octopamine Levels in Leucophaea Maderae

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DEVELOPMENT OF HPLC-EC METHODOLOGY AND ITS USE IN THE DETERMINATION OF THE DAILY PATTERN OF BRAIN OCTOPAMINE LEVELS IN *LEUCOPHAEA MADERAE*

A Thesis

Presented to

the Faculty of the Department of Chemistry

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Bowling Green, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Maya Siddiqui

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DEVELOPMENT OF HPLC-EC METHODOLOGY AND ITS USE IN THE DETERMINATION OF THE DAILY PATTERN OF BRAIN OCTOPAMINE LEVELS IN *LEUCOPHAEA MADERAE*

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DEVELOPMENT OF HPLC-EC METHODOLOGY AND ITS USE IN THE DETERMINATION OF THE DAILY PATTERN OF BRAIN OCTOPAMINE LEVELS IN *LEUCOPHAEA MADERAE*

Maya Siddiqui May 1992 76 pages

Directed by: Darwin B. Dahl, Blaine R. Ferrell and David R. Hartman

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A solid phase extraction technique was developed to aid in removing interfering substances from the cockroach brain extract. Quantitative analysis of octopamine was subsequently achieved by HPLC with amperometric electrochemical detection. The procedure affords 80 to 100 percent recovery of octopamine. The presence of octopamine in brain extract was confirmed by GC-MS.

The daily pattern of brain octopamine levels in cockroach *Leucophaea maderae* was investigated using the HPLC-EC assay procedure. It was concluded that the concentration of brain octopamine in the cockroach *L. maderae* does not exhibit daily rhythm.

CHAPTER I

INTRODUCTION

A. Historical

As early as 1940[1] and 1948 [2], Erspamer[1,2] observed that extracts of posterior salivary glands of *Octopus vulgaris*, when irradiated with ultraviolet light in the presence of air, possessed intense adrenaline-like actions on blood pressure and isolated organs. Erspamer isolated the active constituent of the posterior salivary glands of *Octopus vulgaris* in 1952, determined its identity using two-dimensional paper chromatography to be *l-p*-hydroxyphenylethanolamine, and named it octopamine[3]. Erspamer[3] was not able to detect octopamine in other tissues of the octopus or in extracts from the tissues of vertebrates or other invertebrates.

B. Biological significance of octopamine

The research activities involving octopamine are largely aimed at elucidating biological significance of this compound, in particular its possible involvement in adverse medical conditions of humans and other mammals. The urinary excretion of large amounts of the octopamine metabolite

homovanillic acid (HMVA) in phenylketonurics suggests its role in the disease. Octopamine causes a transient elevation of blood pressure in mammals[1,2]. It has been proposed that the increased level of octopamine acting as a false neurotransmitter causes hepatic encephalopathy[4,5]. Administration of tricyclic antidepressants, in particular iprindol, which acts selectively on the enzyme B monoamine oxidase (MAO), causes an increase in the brain octopamine levels in patients being treated for depression[6]. Octopamine at these increased levels displaces both the noradrenaline (NA), and adrenaline (AD) allowing octopamine to act as a false neurotransmitter. It has been shown that the physiological action of octopamine is only 1/50 as effective as that of NA, and because of this octopamine is thought simply to modify the actions of NA[7]. The insect nervous system has the potential to provide a screening tool for new antidepressant drugs because of the high affinity sodium independent uptake mechanism for octopamine and the potential for subsequent inactivation of any neurally released octopamine.

It has been postulated that the effectiveness of MAO inhibitors used in the lowering of blood pressure is due to the false transmitter action of octopamine in displacing stored NA thereby lowering the NA[8]. NA is implicated in high blood pressure in mammals, including humans. Studies with octopamine have found that the octopamine also acts as a co-transmitter with NA[9]. The function of octopamine in

the vertebrates has yet to be fully ascertained. Octopamine in the vertebrate system coexists with NA in much lower concentration. It is released along with NA, and it may modify the action of this neurotransmitter. It has been detected in the heart, submandibular glands, adrenal glands (9), spleen, and vas deferens(10). A high concentration of octopamine is generally found in the sympathetically innervated peripheral tissues. Octopamine disappears if the tissues are surgically deprived of sympathetic innervation(10) suggesting the source of octopamine from the nervous system. The question surrounding the storage of octopamine has yet to be answered. Octopamine has been found in denervation resistant salivary gland, suggesting that octopamine might be stored in location other than sympathetic nerve endings(11).

In the invertebrates, the octopamine is present in significantly higher concentration. The distribution of octopamine in the cockroach, *Periplaneta americana*, nervous system was studied by Evans et al.(12). The study found the highest concentration of octopamine in the cerebral ganglion (14.68 pmol/piece of tissue), followed by suboesophagial ganglion (5.60 pmol/piece of tissue), prothoracic ganglion (5.28 pmol/pice of tissue), terminal abdominal ganglion (5.26 pmol/piece of tissue), mesothoracic ganglion (4.33 pmol/piece of tissue). A small amount of octopamine is present in the cockroach retina (0.62 pmol/piece of tissue). The amount of octopamine present in the cockroach brain is

similar to the amounts of dopamine and *p*-hydroxytyramine [13]. Octopamine is widely distributed in the cockroach nervous system, and its role has to date not been identified. Nathason and Greengard[14] have demonstrated the role of octopamine as a neurotransmitter in the cockroach. Their study found the existence of a naturally occurring octopamine sensitive adenylate cyclase. Robertson et al.[15] found that octopamine activates phosphorylase in the cockroach nerve cord, suggesting that this glycogenolytic effect might be mediated through an increased cyclic AMP. If this is true, then octopamine acts as a neurotransmitter. The existence of octopamine sensitive adenylate cyclase provides a possible mechanism by which octopamine could be involved in the regulation of carbohydrate metabolism as well as in the physiology of synaptic transmission. The possibility that octopamine levels may vary temporaly can not at this time be ruled out. It is suspected that octopamine has multiplicity of roles, and its effect may be analogous to NA in the vertebrate nervous system. It has been shown by Evans and O'Shea [16], that in the locust, one of the dorsal unpaired medial neurons of the metathoracic ganglion is a octopaminergic neuromodulatory cell. Its function as a neurotransmitter, and a neuromodulator has been established [13, 17, 18]. Saavedra and Brownstein[19] have demonstrated the presence of octopamine in the marine mollusc, *Aplysia californica*, ganglia and in single neurons. The researchers observed

that only the octopamine was present in the neural cells. They noted the absence of catecholamines, which led them to conclude that the octopamine acts as a neurotransmitter in *Aplysia californica*. The study concerning the effects of octopamine on neurones of *A. californica* by Hicks et al.[20] showed that the octopamine containing cell governs the nature of the post-synaptic cell's response. Octopamine elicits an increase of cyclic AMP in the target cell. The possible role of an increased cyclic AMP in the target cell of the locust has not been elucidated. In the locust, the neurons extending along both sides of the insect have been determined to be octopaminergic[21, 22]. Octopamine at low concentration inhibits the contraction rhythm of the muscle. The inhibition of the rhythmic muscle contraction is believed to be mediated by a group of myogenic fibers and is similar to alpha-adrenergic properties. These properties are also noted in the octopamine specific adenylate cyclase in the cockroach brain, and lobster hemolymph. In addition to changes in cyclic AMP, octopamine in the cockroach brain also activates the glycogen phosphorylase [23], subsequently increasing glycogenolysis, resulting in the depletion of large stores of glycogen in the ganglia, most likely from the carbohydrate rich perineurial glial cells[24]. The cockroach brain contains substantial amounts of octopamine which suggests that octopamine plays an important role in the cyclic AMP mediated regulation of glycogenolysis and affords additional evidence that it is a neurotransmitter in

the insect nervous tissue. In study done with lobster, it was found that the nervous system contains no NA and large amounts of octopamine[25]. The peripheral nerves from the lobster thoracic ganglia are rich in octopamine and its synthetic enzymes, including tyramine *beta*-hydroxylase[26]. Octopamine does not appear to be present at the surface of the nerve cells. The cells are not electrically activated by superfusing a high concentration of octopamine, which suggests that the octopamine is present in the root cells, rather than in the nerve ending. Octopamine also stimulates heart rate and beat strength through effects on cardiac ganglion cells, further suggesting its role as neurotransmitter. In firefly lantern, octopamine effects strong luminescence[27, 28]. Because the effects of electrical stimulation of the lantern may be mimicked by phosphodiesterase inhibitors[29], and since it has been shown that the lantern contains octopamine sensitive adenylate cyclase, it has been proposed that octopamine effects are via cyclic AMP.

C. <u>The purpose of this study</u>

In an effort to understand the nature and organization of biological systems, the anatomical distribution and levels of biochemical markers expressed in rhythmic manner in insects and crustaceans have been studied. Insects, in particular the cockroach *L. maderae*, offer an excellent model analogous to the vertebrate system in which to

investigate anatomically localized components of the circadian system.

The circadian system in the cockroach *L. maderae*, is one of the best understood in terms of its anatomical and functional organization. The circadian system that controls the locomotor activity pattern in the cockroach consists of four functionally defined components. These components are two mutually coupled pacemakers that generate the primary timing cue for rhythms, photoreceptors for entrainement, and two coupling pathways, one that mediates the flow of entrainement information from the photoreceptor to each pacemaker and a second that couples each pacemaker to the overt rhythm it controls[30]. The pacemakers in *L. maderae* are situated within the lobula neuropil in each optic lobe. Each pacemaker receives information from photoreceptors in the compound eye. This information resets the clock exactly to a 24-hour period each day. Each of the two pacemakers found in the cockroach optic lobes has three known output (efferent) pathways, which regulate the circadian rhythm of locomotor activity, eye sensitivity to light and metabolism respectively. In a study concerning the level of metabolic activity in the nervous tissue by Lavialle and associates[31] in 1989 for example, they reported a significant change in energy metabolism per 24-hour period. Despite the fact that each of these efferent pathways originate in the lobula neuropil, it is not yet known whether the same cells regulate the expression of each of

these rhythms via efferent neural pathways. The identification of a neurochemical marker that varies on a circadian basis would be an important first step in trying to answer this question. The identification of such a neurochemical marker controlled by the lobula neuropil would enable the discovery of the neurons that produce the neurochemical marker which could then be traced anatomically toward the pacemaker. Octopamine, a prominent neurotransmitter in the cockroach brain offers a likely neurochemical marker candidate. It has been shown that in some arthropods the brain and hemolymph levels of octopamine do vary on a temporal basis[32]. Woodring and associates [32] reported daily rhythm in brain octopamine level in female house cricket *Acheta domesticus*.

As a first step toward determining whether or not octopamine is a potential neurochemical marker useful in tracing neural pathways toward the pacemaker, the purpose of our study will be to determine the existence of variation in brain levels of octopamine in the cockroach *L. maderae*. The study will be conducted at 6-hour intervals throughout a 24-hour period in order to scan for temporal variation. In order to carry out this study, we initially had to develop a highly specific method for the detection of octopamine by high pressure liquid chromatography (HPLC) with amperometric electrochemical detection (EC), previously unreported in the literature. The presence of octopamine in the cockroach

brain was subsequently confirmed by gas chromatography /mass spectrometry.

D. The development of assay methods for octopamine

The occurrence of octopamine was demonstrated in mammalian organs and in human urine after inhibiton of monoamine oxidase (MAO)[32], and using ion-exchange resin to separate selectively, and to concentrate minute amounts of amine for two dimensional chromatography. The enzyme MAO degrades octopamine into *p*-hydroxymandelic acid. It was not until 1968 that octopamine was identified in mammalian tissue by a very sensitive but nonspecific enzyme radiochemical assay (ERA) developed by Axelrod et al. for invertebrates[33]. Octopamine was found, using the ERA technique, among other organs, in several sympathetically innervated organs of the Sprague-Dawley rat[34], and other mammals[32]. The amine has also been detected in human blood plasma and platelets[35]. The technique of Axelrod et al.[33] utilizes the bovine adrenal enzyme phenylethanolamine N-methyltransferase to transfer the methyl group from C^{14}-S-adenosylmethionine to the nitrogen of octopamine to form C^{14}-N-methyloctopamine (synephrine). This enzyme is specific for those phenylethylamines having a *beta*-hydroxyl group. The radiolabeled synephrine formed during the reaction is removed by toluene:isoamyl alcohol solvent extraction at pH 10, and estimated by scintillation counting. A number of biogenic amines are susceptible to N-

methylation under these conditions, and the solvent system commonly used to extract deuterated synephrine from the reaction mixture also extract significant amounts of other amines[36, 37]. Therefore, the identification of octopamine is achieved by the use of thin-layer chromatography. Failure to perform chromatographic checks may account for some of the conflicting reports in the literature concerning the octopamine concentrations in various tissues [38, 39]. It is desirable that chromatographic purification of the [^{3}H]synephrine formed in the octopamine assay be performed routinely on all samples processed through the ERA procedure. The technique of Axelrod et al.[33] determined the concentration of octopamine in the rat brain to be 4.7 ng/g of brain tissue. The largest concentration is found in the adrenal gland amounting to 461 ng/g[33]. The major drawback of the enzymatic assay is the inability to distinguish the three isomers, *ortho*, *para*, and *meta* octopamine[34, 35]. The method of Danielson and associates[40] offers the advantage that the chromatographic procedures used are exhaustive and permit the separation of the N-methyl derivatives of the *meta*- and *para*- isomers of octopamine. Although *meta*-octopamine is not normally detectable in most tissues[41], it constitutes a significant proportion of the octopamine present in the salivary gland[39] and can be detected in the brain after the administration of MAO inhibitor[40]. Although considerably more laborious than other methods, the technique of

Danielson et al.[41] has considerable advantages with regard for specificity that for several years was regarded as the method of choice.

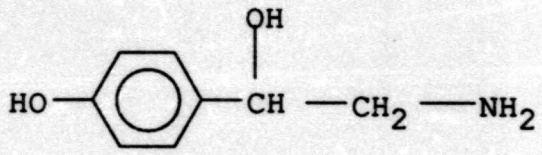
Techniques for the identification of octopamine abound. With the advances made in the field of chromatography, additional techniques have been investigated and developed. Scientists working in the field of gas chromatography (GC), HPLC, and gas chromatography/mass spectrometry (GC/MS) have developed numerous excellent techniques for elucidation of derivatized octopamine. Brooks and Horning [42, 43] describe an excellent method for the estimation and identification of acetylated derivative of octopamine and other biogenic amines, using GC. Edwards and associates [44] in 1972 described a methodology for the identification of the O-trimethylsilyl (O-TMS), N-dinitrophenyl (N-DNP) derivative of octopamine on GC with Electron Capture Detector (ECD). They report the detection of octopamine in rat brain and liver in picogram levels. Buck and colleagues[45] in 1977 described an assay method for octopamine based upon GC/MS. Octopamine, extracted from tissues with perchloric acid, is purified by column chromatography on Dowex 50, derivatized with pentafluoro propionic acid anhydride (PFPAA), and separated by gas chromatography on OV-17 prior to mass spectrometric analysis. Although less sensitive than the radiochemical methods, this technique is capable of providing valuable corroboration of results obtained with more sensitive but

less definitive radiochemical procedures. Bailey et al.[46] in 1982 described a methodology for the identification of octopamine using (HPLC) with amperometric electrochemical detection. The procedure uses a silver/silver chloride (Ag/AgCl) reference electrode, which was subject to drift due to the diffusion of chloride ions from the reference electrode into the mobile phase. Difficulties encountered with amperometric detection has kept the development of methodologies nonexistent to date. Bailey and colleagues [46] reported using an oxidation potential of 0.9 V vs Ag/AgCl electrode to determine octopamine levels; however they did not generate cyclic voltammogram for octopamine. The authors state that due to the high oxidation potential needed to oxidize octopamine, amperometric detection is not an attractive method to quantitate octopamine. In 1983, Martin and associates[47] developed a method using HPLC with coulometric electrochemical detection. The authors report that oxidizing the mobile phase prior to injection of the sample reduces significantly the background noise, and thus enhances the sensitivity of the methodology. In 1984, Ibrahim et al.[48] reported a method for quantitative measurement of octopamine using capillary column gas chromatography electron capture negative chemical ionization (NCI) GC/MS. They reported the existence of the three isomers of octopamine in human urine. The deuterated amine was subjected twice to cation exchange chromatography, using 5 cm x 0.8 cm column of AG 50W - X2 ion-exchange resin. The

resultant octopamine fraction was derivatized with (PFPAA), and under electron capture NCI GC/MS, the PFPAA derivatives gave M^- and $(M-HF)^-$ ions which were sufficiently abundant to be suitable for selected ion monitoring. The published methodologies for the detection, quantification, and identification of octopamine most commonly use NCI GC/MS by electron capture[49, 50]. To date there is not one universal method for octopamine determination.

E. Chemistry of Octopamine

Octopamine is an aromatic primary monoamine, it is an alcohol, and is named as *beta*-hydroxyphenylethanolamine. The structure of octopamine is shown below:



Octopamine

The presence of a *beta*- hydroxy group makes octopamine unstable at high temperature. The chemical bond between the

nitrogen and carbon is easily broken. A naturally occurring form of octopamine is the D- form. Octopamine is believed to be synthesized *in vivo* by way of phenylalanine. The enzymes responsible for the biosynthesis of octopamine are phenylalanine 4-hydroxylase (PAH), aromatic L-amino acid decarboxylase (AADC), and dopamine- *beta*- hydroxylase (DBH)(51). There are several possible pathways for these reactions to occur. Examples of choices available for octopamine synthesis from phenylalanine are: via tyrosine, tyramine, octopamine pathway, or phenylalanine, phenylethylamine, tyramine, octopamine pathway. It could also be synthesized from the dehydroxylation of catecholamines dopamine and noradrenaline by DBH enzyme. These reactions are shown in Figure 1.

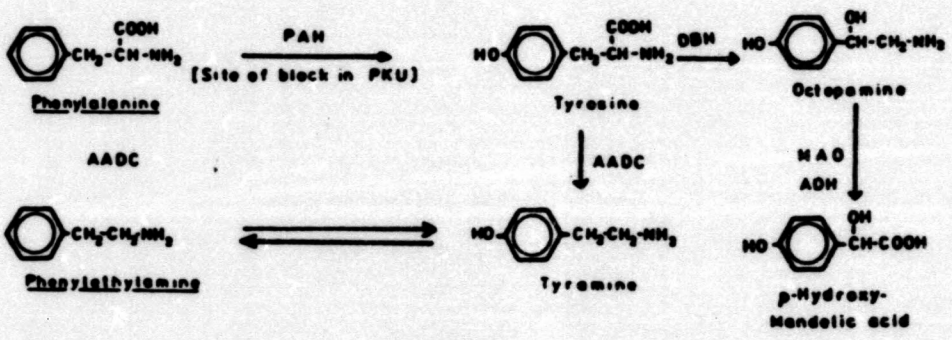


FIGURE 1. Biosynthesis and Degradation of Octopamine

In vertebrates, octopamine is metabolized by monoamine oxidase (MAO) enzyme to *p*-Hydroxymandelic acid. Studies have shown that MAO inhibitors produce a rise in octopamine levels in tissue [52], indicating the importance of this enzyme in the normal regulation of octopamine concentration.

CHAPTER II

EXPERIMENTAL

A. Instrumentation

Varian HPLC Model 5000 with a 25 µL injection loop, and Varian 4270 Integrator were obtained from Varian Analytical Instruments, 220 Humboldt Ct., Sunnyvale, CA 94089, USA. BAS Model 100 electrochemical analyzer, BAS LC-4B/17A amperometric detector, glassy carbon electrode and Ag/AgCl reference electrode were purchased from BAS Bioanalytical Systems Inc., 20701 Kent Ave., West Laffayette, IN 47906, USA. A Visiprep™ SPE vacuum manifold, complete with adapters used in the solid phase extraction, Supelcosil LC-18 HPLC column, and 1 mL Supelcosil NH_2 solid phase extraction columns were purchased from Supelco, Inc., Supelco Park, Bellefonte, PA 16823-0048, USA.

The gas chromatography (GC) system was HP 5890 from Hewlett-Packard, Rte. 41, P. O. Box 1100, Avondale, PA 19311, USA. The GC column was a Rt_x-5, 20m x 0.23mm, 0.1 microns, df from the Restek Corp., 110 Benner Cir., Bellefonte, PA 16823, USA. The mass spectrometer system was a Kratos Concept IH/Double Focusing EB Geometry EI, from Kratos Analytical, Barton Dock Rd., Urmston, Lancashire M31 2LD, England.

B. Materials

Methylene chloride HPLC GC/MS Grade and concentrated phosphoric acid were obtained from Fisher Scientific, 711 Forbes Ave., Pittsburgh, PA 15219, USA. Acetonitrile HPLC Grade, methyl alcohol HPLC Grade, 3,4-dihydroxybenzylamine hydrobromide (DHBA), 1-heptanesulfonic acid sodium salt, and N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Aldrich Chemical Co., Inc., P.O. Box 355, Milwaukee, WI 53201. The DL-*p*-octopamine hydrochloride, ethylenediaminetetrahydroacetic acid (EDTA), free acid, and potassium phosphate dibasic were purchased from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA. Helium carrier gas was supplied by the Central Welding Supplies, Lexington, KY 40205, USA. Acetic anhydride, acetic acid, methanol, ethyl acetate, all ACS grade were purchased from the J&W Scientific, 91 Blue Ravine Rd., Folsom, CA 95360 USA. A C_{18} reverse phase 25 cm x 21 mm with 5 micron particle size column, and 1 mL LC-NH_2 Supelclean solid phase extraction cartridges were purchased from Supelco Inc., Supelco park, Bellefonte, PA 16823, USA.

C. Procedure

1. *Preparation of the mobile phase.* The mobile phase was prepared by dissolving 8.7090 g of K_2HPO_4 in about 400

mL of HPLC grade water into which 0.100 g of EDTA was then added and allowed to dissolve completely. Sixty mL of HPLC grade methanol was added. The solution was thoroughly mixed. The volume was brought up to about 950 mL with HPLC grade water. The pH of the solution was adjusted to 6.5 with concentrated phosphoric acid. Then the volume was brought up to the one liter mark. At this point, about 50 mL of the mobile phase was withdrawn for use in the brain extraction. Then 0.30 g of 1-heptane sulfonic acid was added to the remaining 950 mL of mobile phase. The mobile phase was then allowed to stand for 10-12 hours prior to HPLC analysis.

Following the preparation of the mobile phase, the HPLC instrument was powered on and a solution of 80/20 methanol/water mixture was run through the column for 10 -12 hours. Prior to use, the mobile phase was filtered through a 0.45 μm Nylon 66 membrane filter and degassed for a minimum of 15 minutes. At the end of twelve hours, the methanol/water mixture was removed from the HPLC and replaced with previously degassed mobile phase containing the ion-pair reagent (heptane sulfonic acid). The flow rate was adjusted to 0.6 mL/min and the column temperature was set at 30^{o}C. The mobile phase was allowed to run for 10-12 hours to allow the column packing to equilibrate with the mobile phase. At the end of this time, the detector was turned on, the reference and the working electrodes were positioned in place and connected, the sensitivity was set

to 0.5nA, and the voltage was set at 1.040 V vs the Ag/AgCl reference electrode. The detector was operated in an oxidation mode. The electrodes were allowed to equilibrate for about 30 minutes to 3 hours, until a steady baseline was achieved. Octopamine standard containing 0.4 ng of octopamine/µL was prepared by carefully weighing out 2.5 mg of octopamine and dissolving it in a 25 mL volumetric flask using the mobile phase containing the heptanyl sulfonic acid. Next, a 100 µL of this solution was withdrawn and diluted in another 25 mL volumetric flask. This solution was then called octopamine solution Number 2. The 0.64 ng/µL DHBA standard was prepared by carefully weighing out 4.0 mg of DHBA and dissolving it in 25 mL mobile phase containing heptane sulfonate, then withdrawing 100 µL of this solution and diluting it into another 25 mL volumetric flask. This became DHBA solution Number 2. The sensitivity of the detector was determined each time a new mobile phase was prepared. This was achieved by preparing the 0.4 ng/µL and the 20 ng/µL of octopamine standards and injecting 25 µL of each solution to evaluate the suitability of the obtained peak heights for octopamine in the cockroach brain extract. The 0.4 nanogram octopamine standard was prepared by taking 2 mL of octopamine solution Number 2 and diluting it to 10 mL with the mobile phase. Then 500 µL of this solution was mixed with the 500 µL of DHBA standard solution Number 2. Just prior to the surgery, the standard containing 0.64 ng of DHBA was prepared using the mobile phase not containing

ion-pair reagent to facilitate the octopamine extraction, by weighing out 4.0 mg of DHBA and dissolving it in 25 mL volumetric flask. One hundred µL of this solution was withdrawn and placed into another clean 25 mL volumetric flask. The volume was brought up to the 25 mL mark. The flask containing 0.64 ng of DHBA/µL was then taken to the surgery room.

2. Electrochemical behavior study. Initial work was done to determine the electrochemical behavior of octopamine and DHBA. This was achieved by generating the cyclic voltammogram for each compound. The cyclic voltammogram was obtained by using the BAS 100 instrument, the sensitivity of which was set at 1.0 E^{-4} (A/V). Ag/AgCl reference electrode and glassy carbon working electrode were used. Octopamine and DHBA were prepared by dissolving 28.4 mg of octopamine and 33.0 mg of DHBA in 50 mL of mobile phase respectively. About 10 mL of each solution was withdrawn from the flask and placed in the electrolysis cell and bubbled with nitrogen for five minutes. The potential scan was from 0.0 mV to 1100 mV and back to 0.0 mV at a rate of 250 mV/sec. The result is shown in Figure 2. The cyclic voltammogram for DHBA standard was run from -50 mV to 1500 mV to -50 mV as shown in Figure 3.

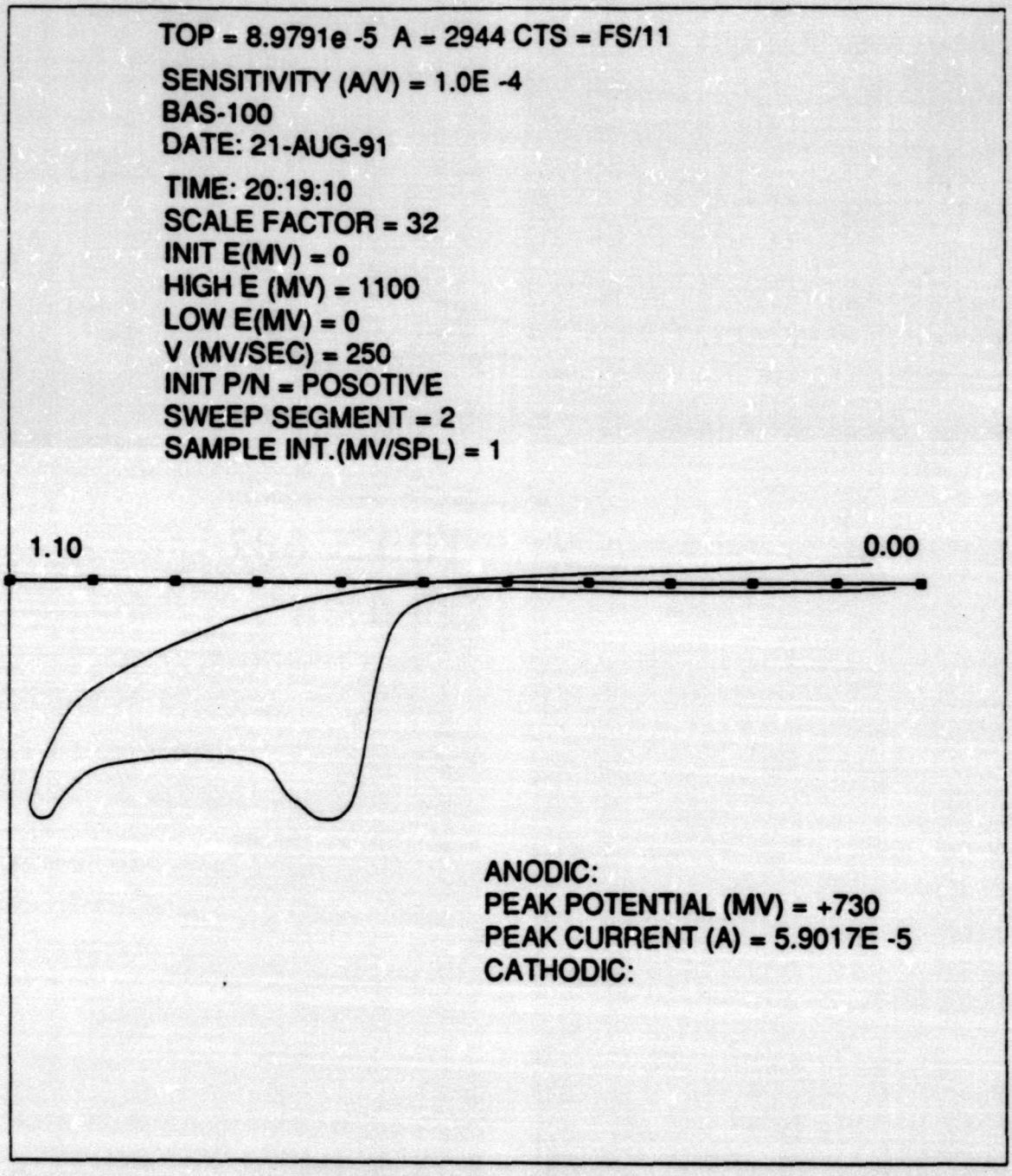


Figure 2. Cyclic voltammogram for octopamine.

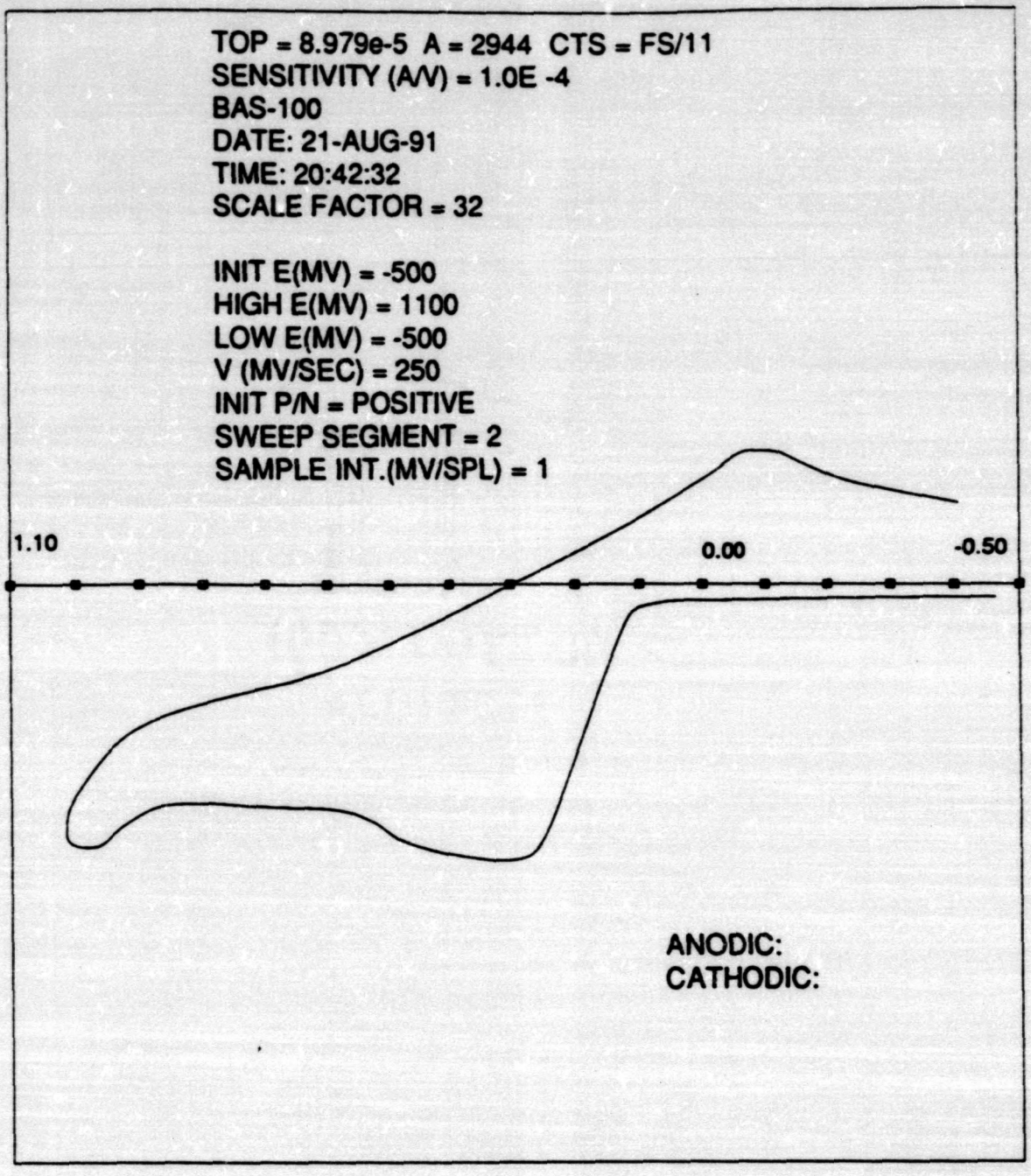


Figure 3. Cyclic voltammogram for internal standard DHBA.

A solution containing a 50:50 mixture of 0.04 ng/µL standard octopamine and 0.06 ng/µL standard DHBA was used in generating the hydrodynamic voltammogram. The generation of the hydrodynamic voltammograms for octopamine and DHBA used HPLC with amperometric electrochemical detection. The range was from 100 mV to 1100 mV vs Ag/AgCl. The electrodes were allowed to equilibrate between each successive downward adjustment of the output voltage. A plot of the current generated vs the applied potential is shown in Figure 4. Each point on the graph represents an average of three successive injections of 25 µL of the octopamine:DHBA standard mixture described above. A value of 1.040 V was chosen from the graph in Figure 4 as the optimum operating potential for our electrochemical detector.

The limit of detection for octopamine used in our study was determined by increasing the sensitivity of the detector to the point where a measurable background was obtained. Octopamine standard was injected followed by a subsequent dilution until the ratio of peak height of the octopamine and the background signal was approximately equal to 3. Figure 5 shows a chromatogram of an injection of 70 pg of octopamine giving a signal to noise (S/N) ratio of approximately 3.

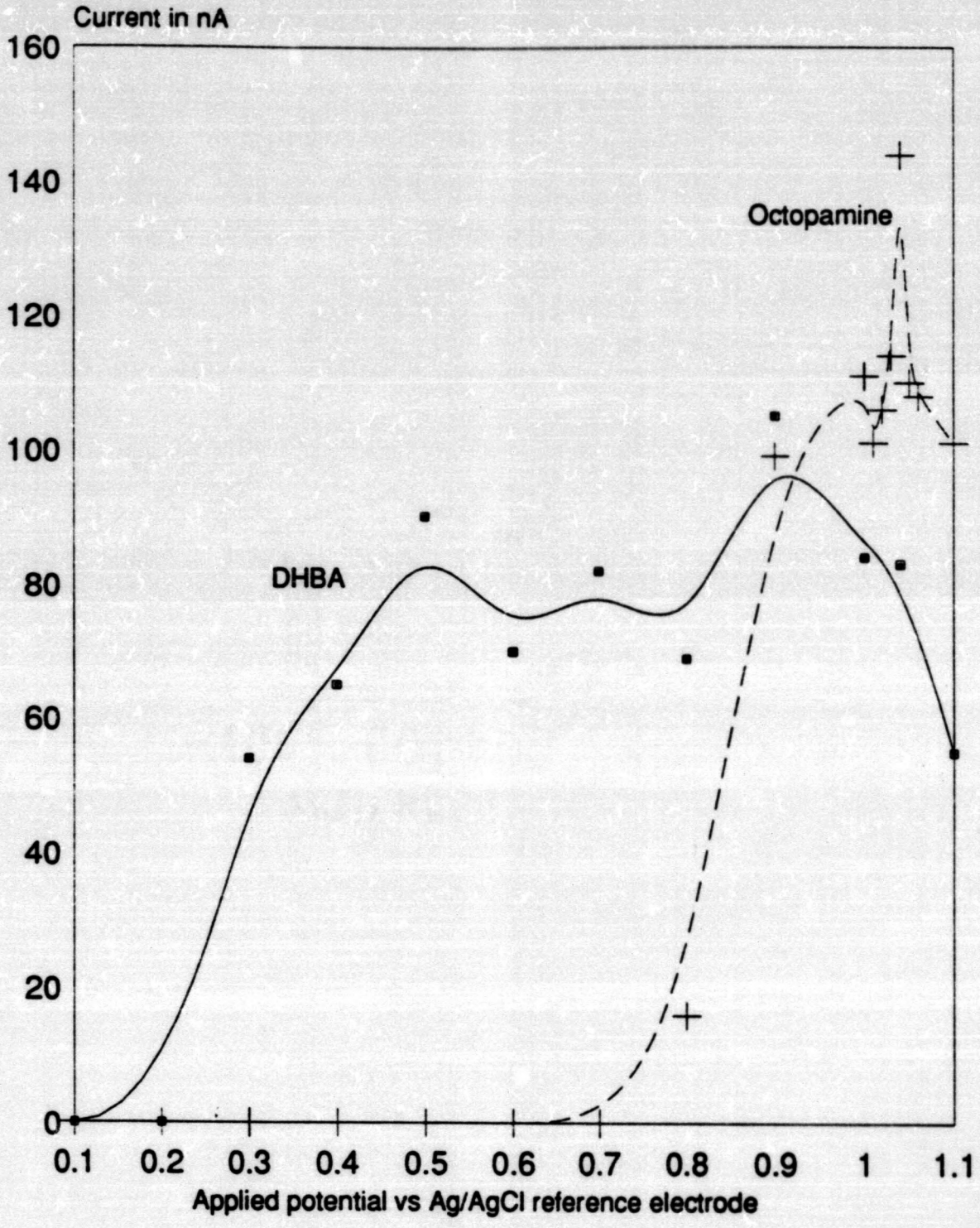


Figure 4. Hydrodynamic voltammogram for standard octopamine and DHBA (internal standard).

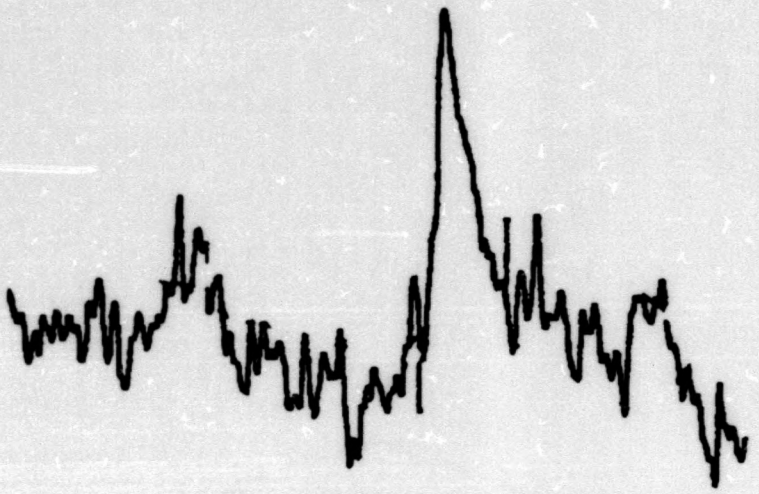


FIGURE 5. HPLC Chromatogram illustrating limit of detection. The detector was set at 0.05 nA sensitivity.

3. Cockroach brain preparation and liquid-liquid extraction. The experimental cockroaches *L. maderae* were initially raised in the controlled environment under the 12:12 light/dark cycle, and ambient room temperature. At least one week prior to surgery, the cockroaches were acclimatized to 25° ± 2° C temperature, and 12:12 light/dark cycle environment within the environmental chambers. Light onset was at 0800 h. During this time, the daily activity,

as shown in Figure 6, was recorded for representative cockroaches as during the acclimation period.

Brain extracts were obtained over a 24-h period at 6-h intervals, beginning at 0800 h and ending at 0200 h, giving four readings of octopamine levels in the cockroach brain. Immediately prior to the surgery, each cockroach was removed from the plastic holding cage within the environmental chamber and flash frozen in -70° C petroleum ether. This method minimized trauma to the insect thus avoiding a possible stress induced rise in hemolymph levels of octopamine. The head of the cockroach was then placed through a hole cut in the lid of a plastic petri dish. Tape was placed behind the head to maintain its position. The lid was placed over the petri dish base and the dish positioned on the illuminated stage of a dissecting microscope. An incision was made through the cuticle of the head capsule encompassing both antennae, using a fractored razor blade scalpel. The cuticle was removed, exposing the brain. The left optic nerve was severed using iridectomy scissors, and the brain lifted out of the head enclosure. The right optic nerve was cut at this time to free the brain. The brain was cleaned of any excess fat and carbohydrates prior to its removal. Two brains were placed into an Elrejhen tissue homogenizer to which 100 µL 0.64 ng of DHBA/µL, 50 µL of 0.1 N perchloric acid, ($HCLO_4$), and 50 µL of the mobile phase without heptane sulfonic acid was added. The pooled brains were then homogenized for 30 sec.

Test Date: February 22 - March 6, 1992

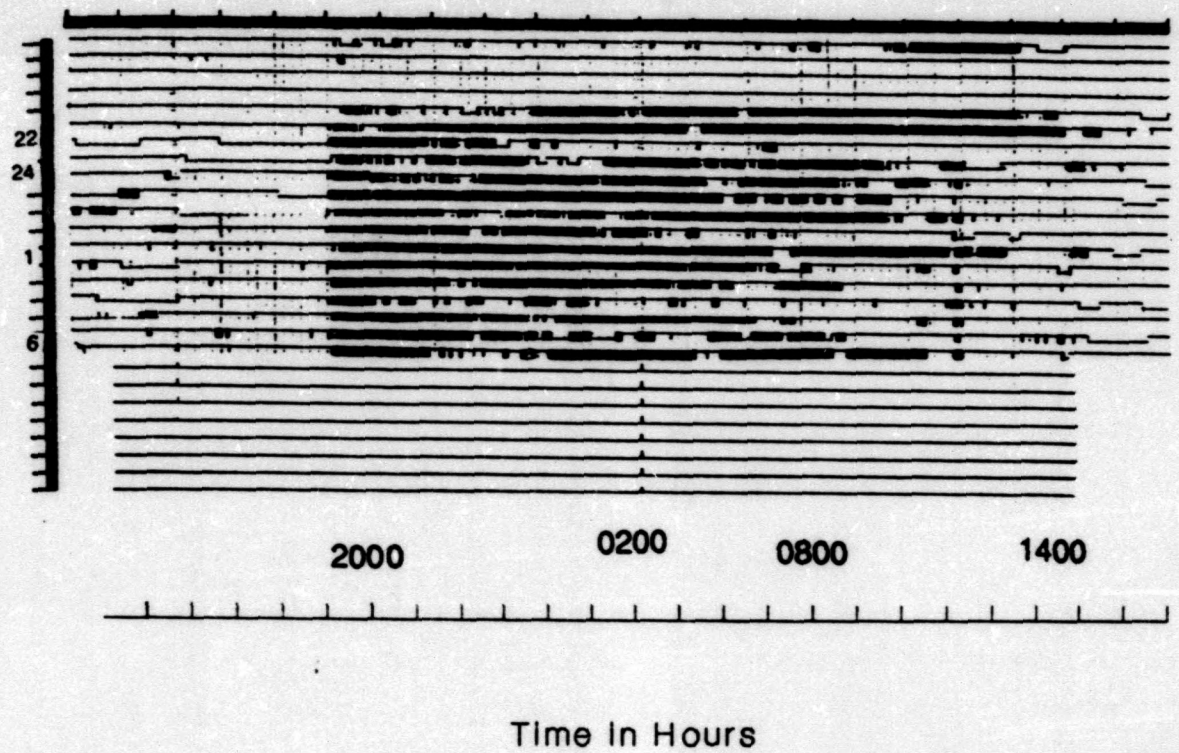


Figure 6. Cockroach activity chart.

The homogenate was transferred to a microcentrifuge tube and centrifuged at 4° C for 30 min at 20,200 x g. A micropipette was used to transfer the supernatant from the microcentrifuge tube to transfer it to another clean microcentrifuge tube. The pellet was discarded. One mL of 8:1 heptane:chloroform (v/v) mixture was added, and the solution vortexed for 30 sec. The aqueous bottom layer was transfered to another clean microcentrifuge tube using a 1cc tuberculin syringe. Five µL of this extract was analyzed by HPLC. Figure 7 shows a representative chromatogram of brain extract under the described conditions.

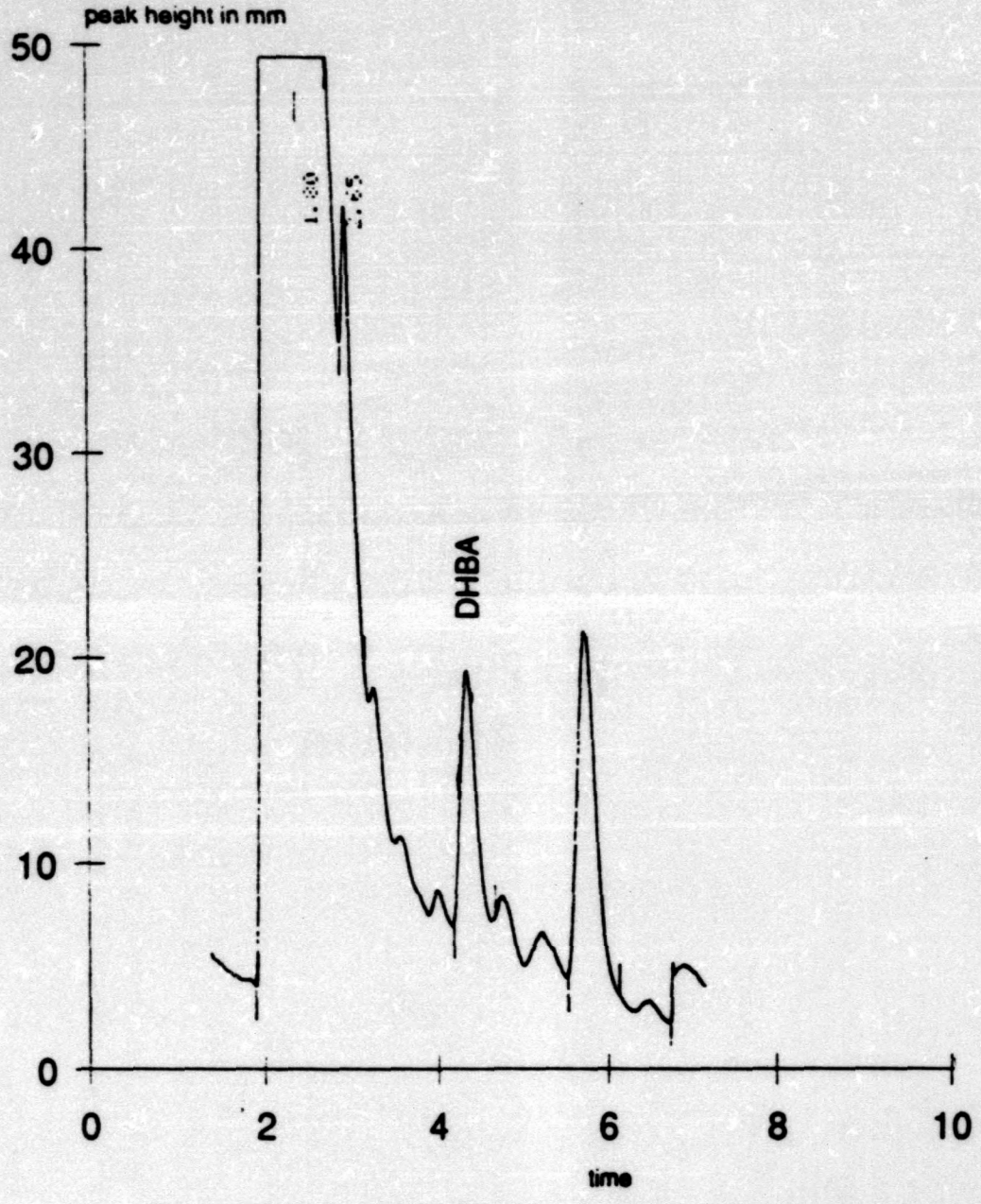


Figure 7. HPLC-EC chromatogram of 5 µL of brain extract without the use of LC-NH_2 SPE column.

4. Method development using solid phase extraction for quantification of brain octopamine in L. maderae. As can be seen from Figure 7, in order to quantify and subsequently identify the octopamine in the cockroach brain, it was necessary to develop a suitable clean up method for the determination of octopamine in the cockroach brain. It was found that NH_2 solid phase extraction columns using 50:50 (v/v) methylene chloride: methanol as a conditioning solvent worked best, the result being shown in Figure 8.

As part of our method development, it was necessary to determine the optimum elution volume to use to elute the sample. A 100 µL of 0.4 ng/µL of the octopamine standard was allowed to pass through the conditioned column followed by washing the sample one time with 100 µL of the mobile phase. The collected eluent was analyzed by HPLC with amperometric detection for the presence of octopamine. A second sample of 100 µL of the above octopamine standard was put on the conditioned LC-NH_2 column and washed twice with 100 µL of the mobile phase. The eluent was analyzed for the presence of octopamine. The procedure was repeated until the optimum wash volume was determined by calculating the amount of octopamine present in each of the eluates, using a standard calibration curve. The percent recovery study was performed by constructing an octopamine standard curve without the use of the solid phase extraction column. A linear regression analysis was performed on the data. Then 100 µL of the 0.4 ng/µL octopamine standard was passed

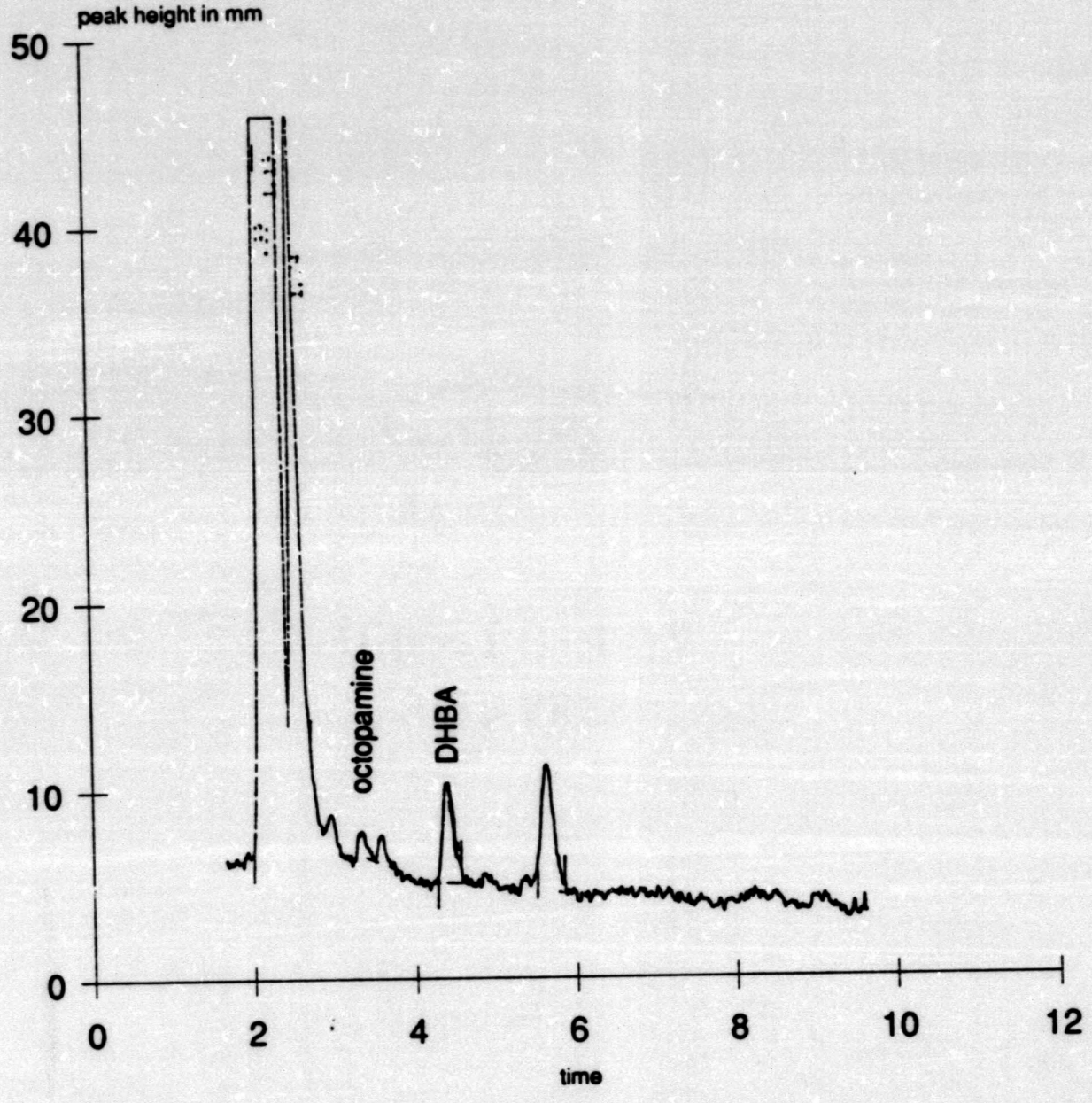


Figure 8. HPLC chromatogram of 25 µL injection of brain extract after the clean-up wtih LC-NH_2 SPE column.

through the conditioned solid phase extraction column, and the percent recovery for octopamine was calculated. A linear regression analysis was used to establish the values for the equation of straight line from which the results were subsequently obtained. The elution volumes and corresponding recovery of octopamine from the NH_2 column is shown in Table 1. The results in graphic form are shown in Figures 9 and 10. The optimum elution volume was found to be 400 µL.

TABLE 1

Percent recovery of octopamine from LC-NH_2 SPE columns using different elution volumes

Wash Volume in mL	Peak Height in mm	Concentration		% Recovery
		Actual in ng	Measured in ng	
0.00	0.00	40.00	0.00	0.00
100.00	0.00	5.00	0.00	0.00
200.00	29.00	3.30	0.47	4.00
300.00	64.00	2.50	1.05	42.00
400.00	120.00	2.00	1.97	99.00
500.00	97.00	1.70	1.59	94.00
600.00	83.00	1.40	1.36	97.00

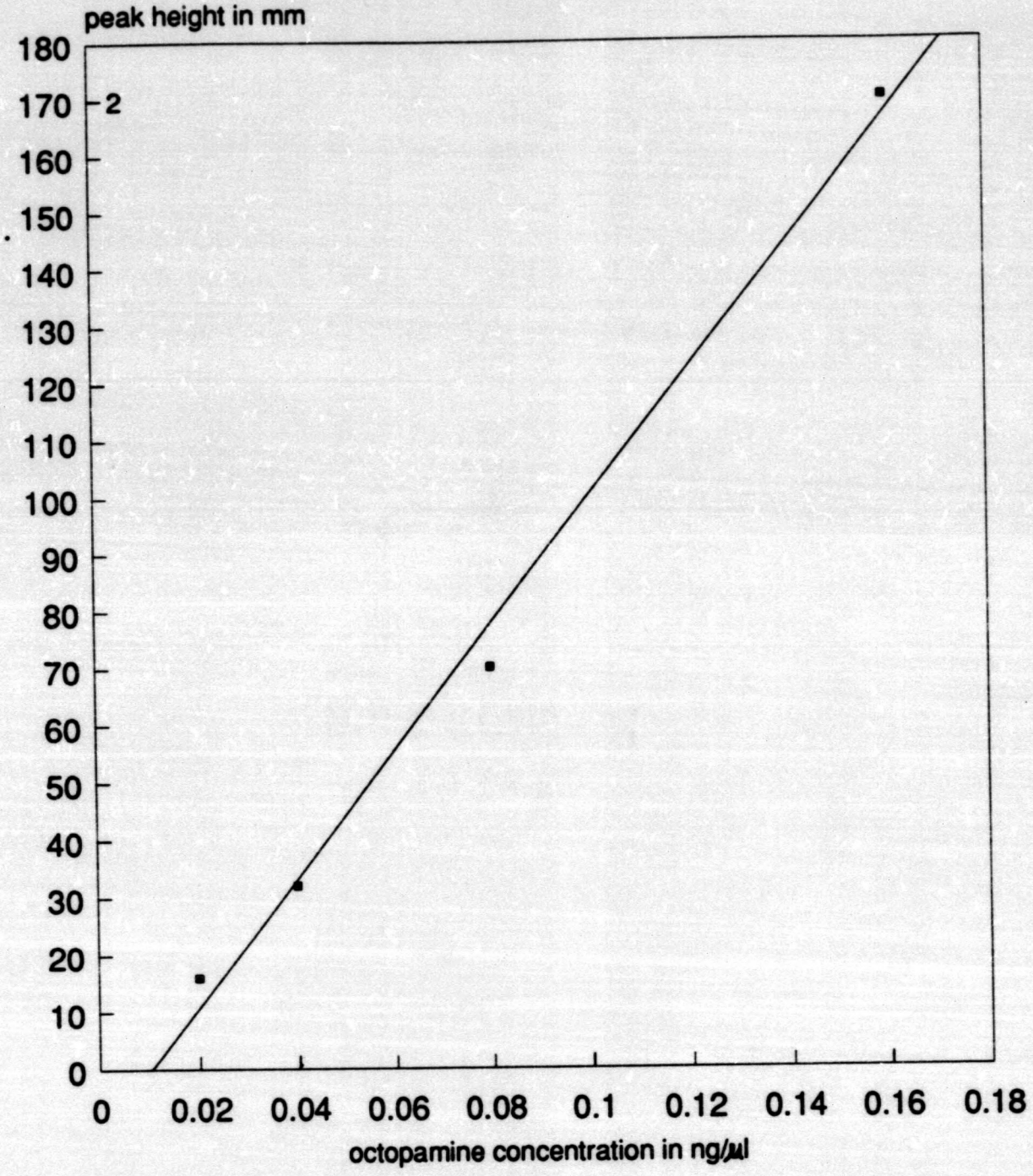


Figure 9. Octopamine standard curve for optimum wash volume.

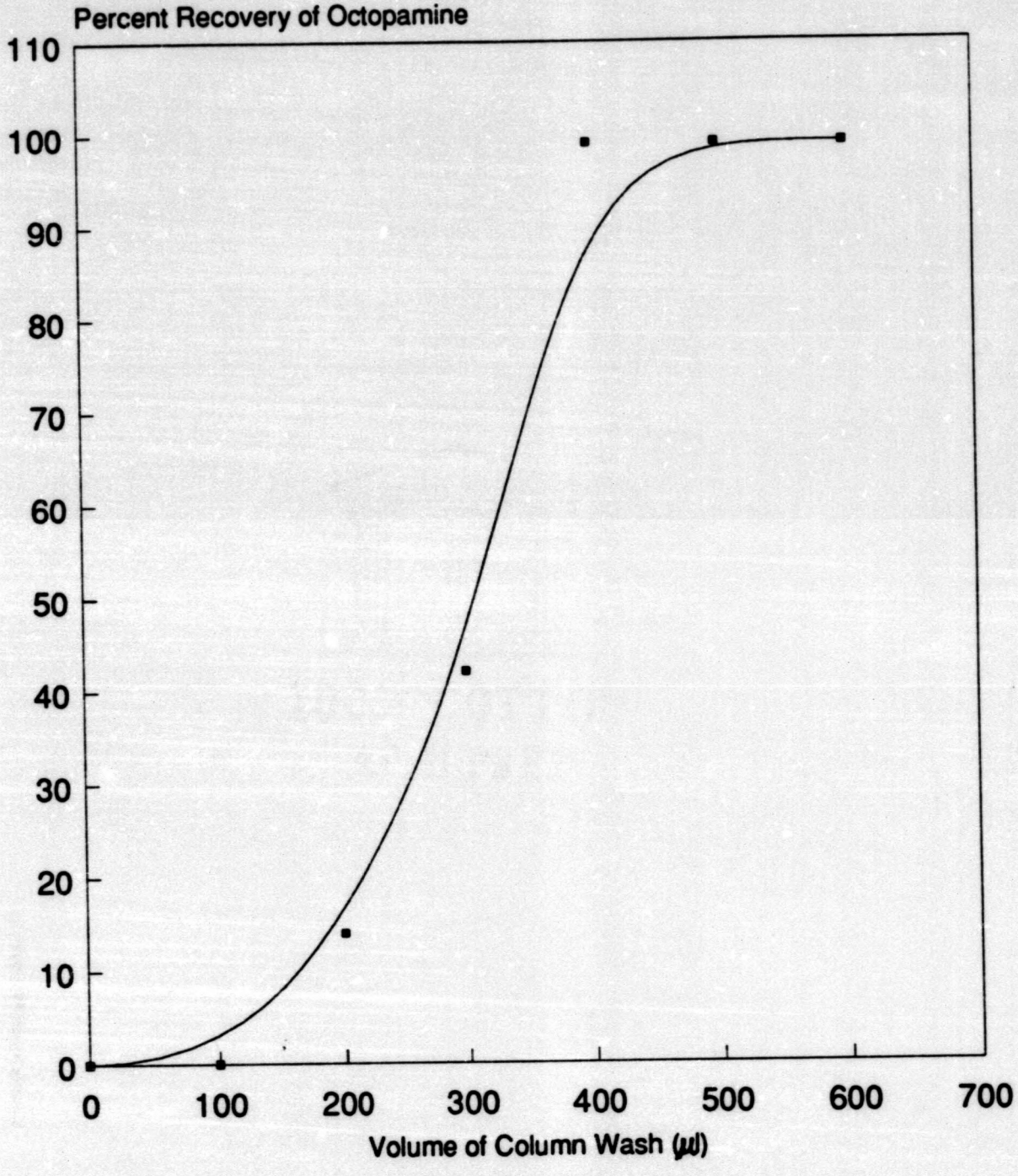


Figure 10. Optimum elution volume for the recovery of octopamine form LC-NH_2 SPE column.

For percent recovery of DHBA, the same elution volumes were used as were for octopamine. The DHBA standard curve was constructed as shown in Figure 11, then the 0.32ng/μL DHBA standard was passed through the LC-NH_2 SPE column. The percent recovery for DHBA was around 98 percent as is shown in Table 2. Upon establishing optimum elution volumes for octopamine, it was found that using one LC-NH_2 SPE column did not give completely satisfactory results with the brain extract. Therefore the entire volume of the eluted sample from one conditioned LC-NH_2 column was passed through a second conditioned LC-NH_2 SPE column. This time, the sample was allowed to completely run off the solid phase packing without an additional wash. The recovery for the second column was about 79-80 percent for DHBA and 80-82 percent for octopamine. The results are shown in Table 2.

TABLE 2

Recovery of octopamine and DHBA using two LC-NH_2 SPE columns in sequence.

Eluting compound	% recovery LC-NH_2 SPE column	
	First column	Second column
Octopamine	97 - 99	80 - 82
DHBA	98 - 100	79 - 80

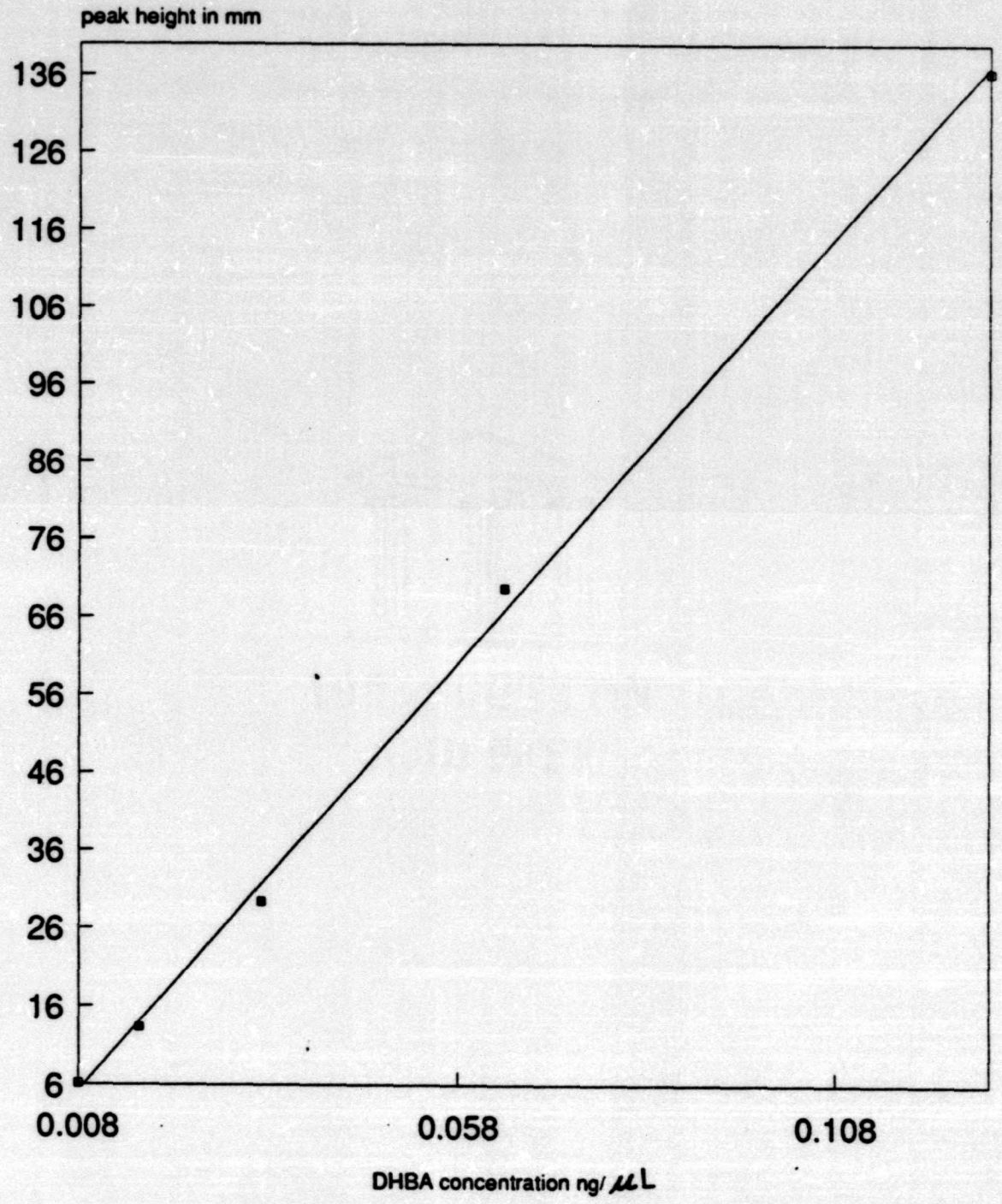


Figure 11. DHBA standard curve.

Brain extracts and standards were treated the same way. One mL NH_2 solid phase extraction cartridge was conditioned with exactly 0.5 mL of 50 : 50 mixture of methanol : methylene chloride (CH_2Cl_2), followed by 1 mL HPLC water and 2mL of the octopamine mobile phase, respectively. The washings were discarded. One hundred µL of the pooled brain extract from two cockroaches was passed through the first column down to the first frit line marked on the cartridge as shown in Figure 12, followed by a subsequent washing with 400 µL of mobile phase. The eluent was collected into a clean microcentrifuge tube, and passed through a second conditioned NH_2 solid phase extraction cartridge this time all the way through past the second frit line. Twenty-five microliters of the collected eluent was then injected on to the Supelcosil LC_{18} HPLC Reverse Phase Column, and detected using the amperometric electrochemical detector. Each set of specimen was injected in triplicate, followed by a triplicate injection of one of the three levels of octopamine / DHBA standard. The chromatographic results are shown in Figures 13, 14. At the end of each of the 24 hour runs, peak heights of octopamine and that of DHBA in the standard and in the cockroach brain were carefully measured and recorded. The three values were averaged, and the resulting values of peak height of octopamine / peak height of DHBA ratio vs the concentration of standard octopamine was used to calculate the octopamine levels in the cockroach brain.

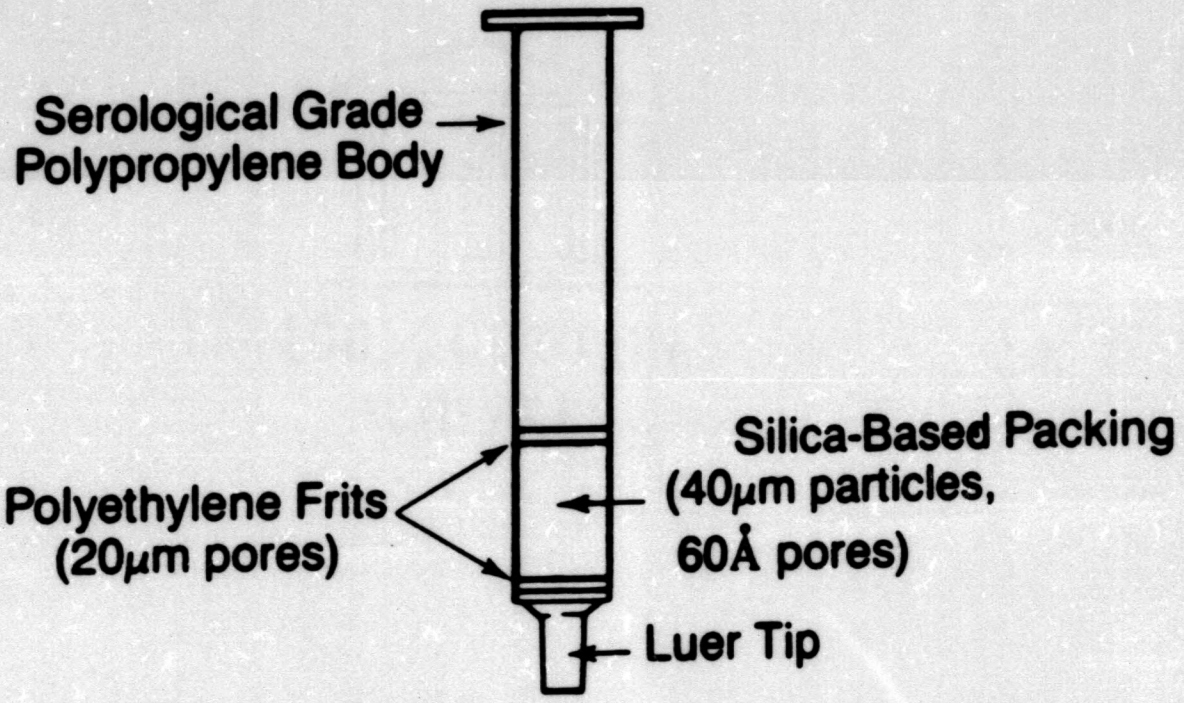


Figure 12. Illustration of an SPE column.

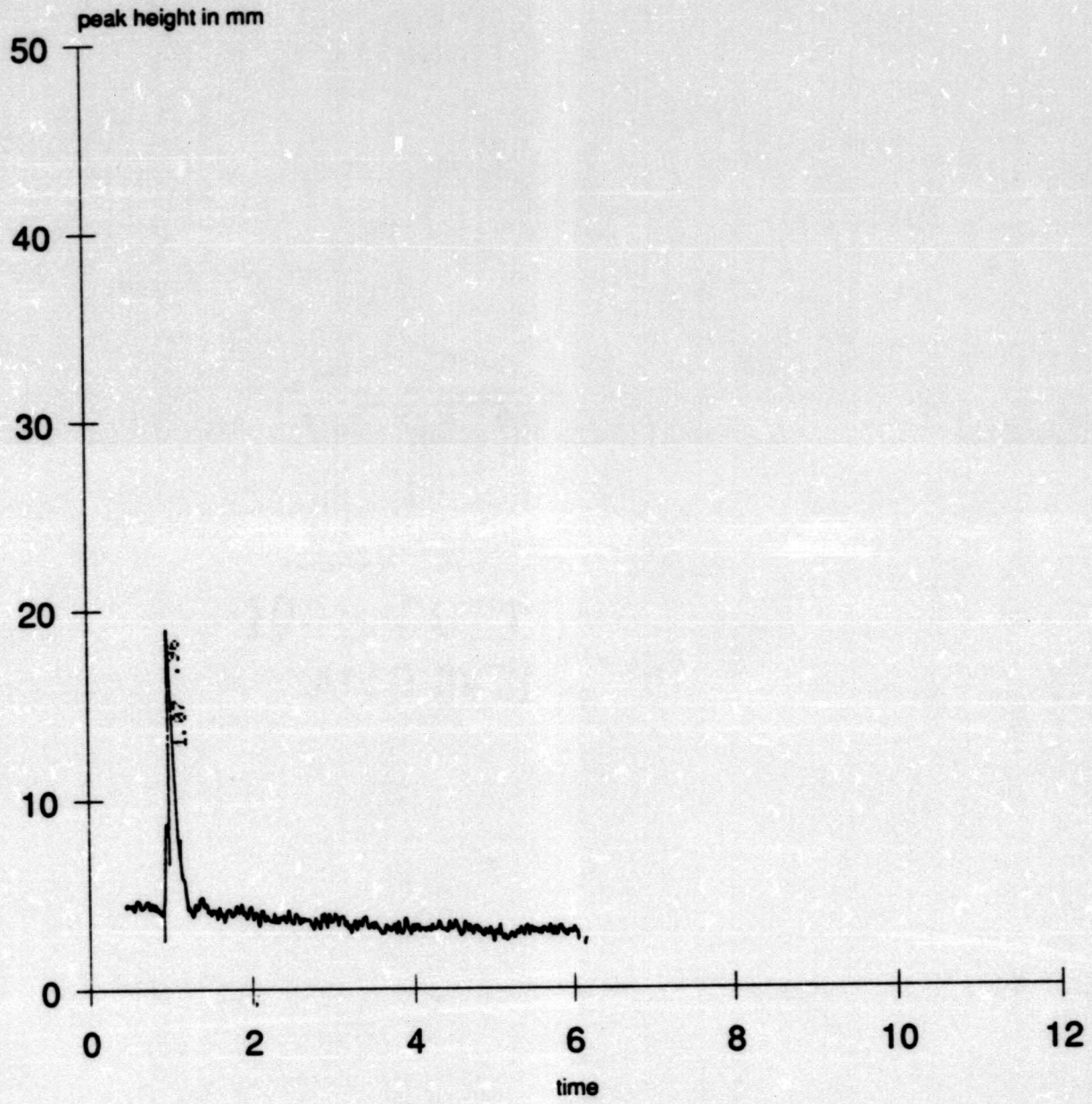


Figure 13. HPLC chromatogram of a good baseline as part of quality assurance.

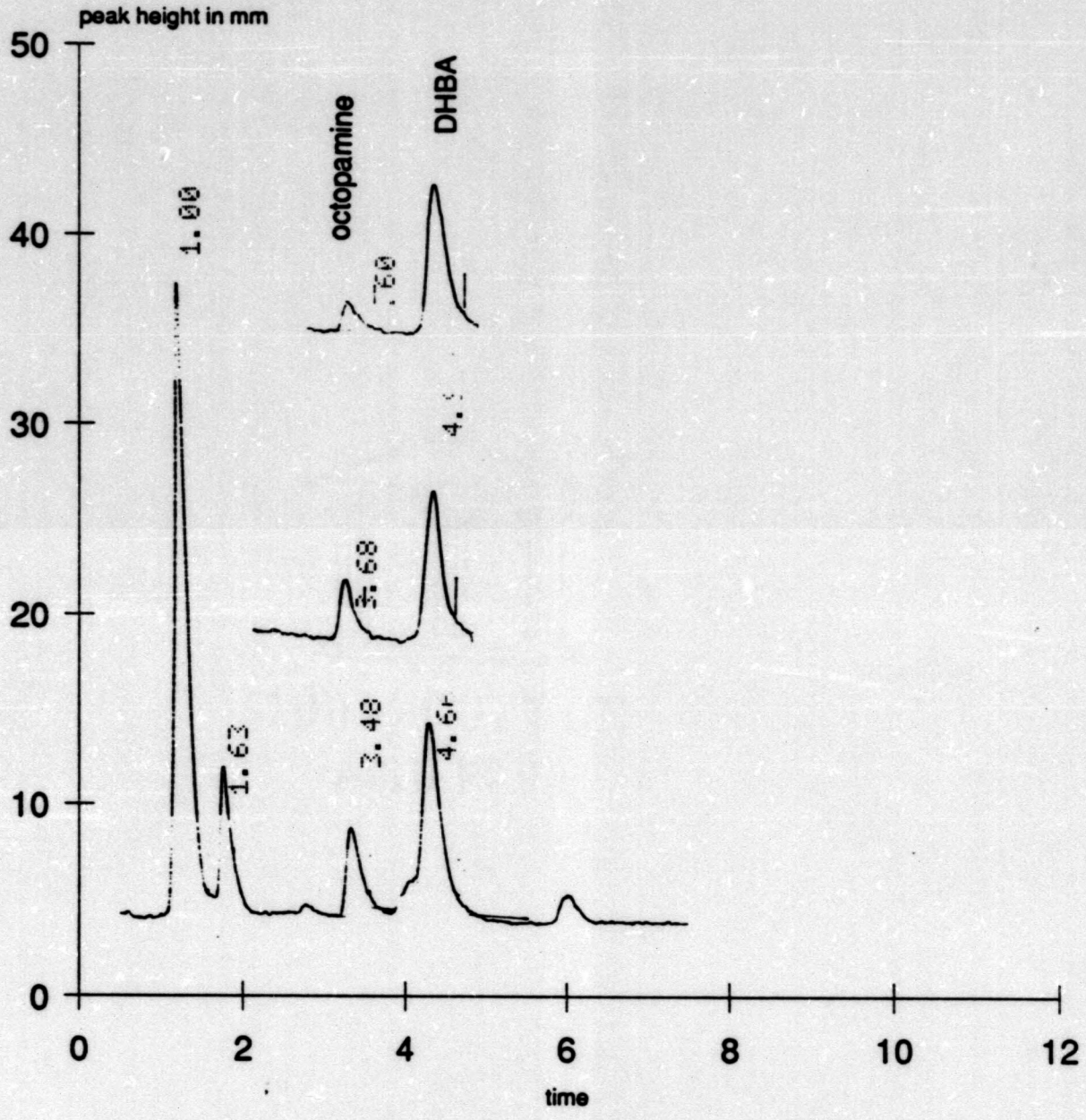


Figure 14. HPLC chromatograms of the octopamine and DHBA standards used to construct the standard curve.

The data was subjected to a regression analysis, followed by the construction of the standard curve which is shown in Figure 15. The concentration of octopamine in the cockroach brain was calculated by dividing the endogenous octopamine peak height by the internal standard DHBA peak height and using this value in the straight line equation obtained from the linear regression analysis. An example of the calculation is shown below: Equation for the straight line is $Y= MX + B$ where Y is the ratio of the octopamine/DHBA peak height, M is the slope of the line, and B is the Y intercept when there is no octopamine present. Therefore it follows that the amount of octopamine (X) present in the cockroach brain is: $X = (Y - B)/M$. B and M are obtained from the regression analysis. Y is obtained from the ratio of peak heights of the octopamine and the internal standard DHBA. The weighted standard deviation was first calculated for each of the four data sets (LN1-5, MD1-5,LF1-5, and MN1-5) of the five REPS, and then for the five data sets representing the REPS 1-5, and four groups (LN1, MD1, LF1, MN1, followed by LN2, MD2, LF2, and MN2 etc.,) until all five REPS were represented. The weighted standard deviation for infinite numbers of data sets was calculated as follows:

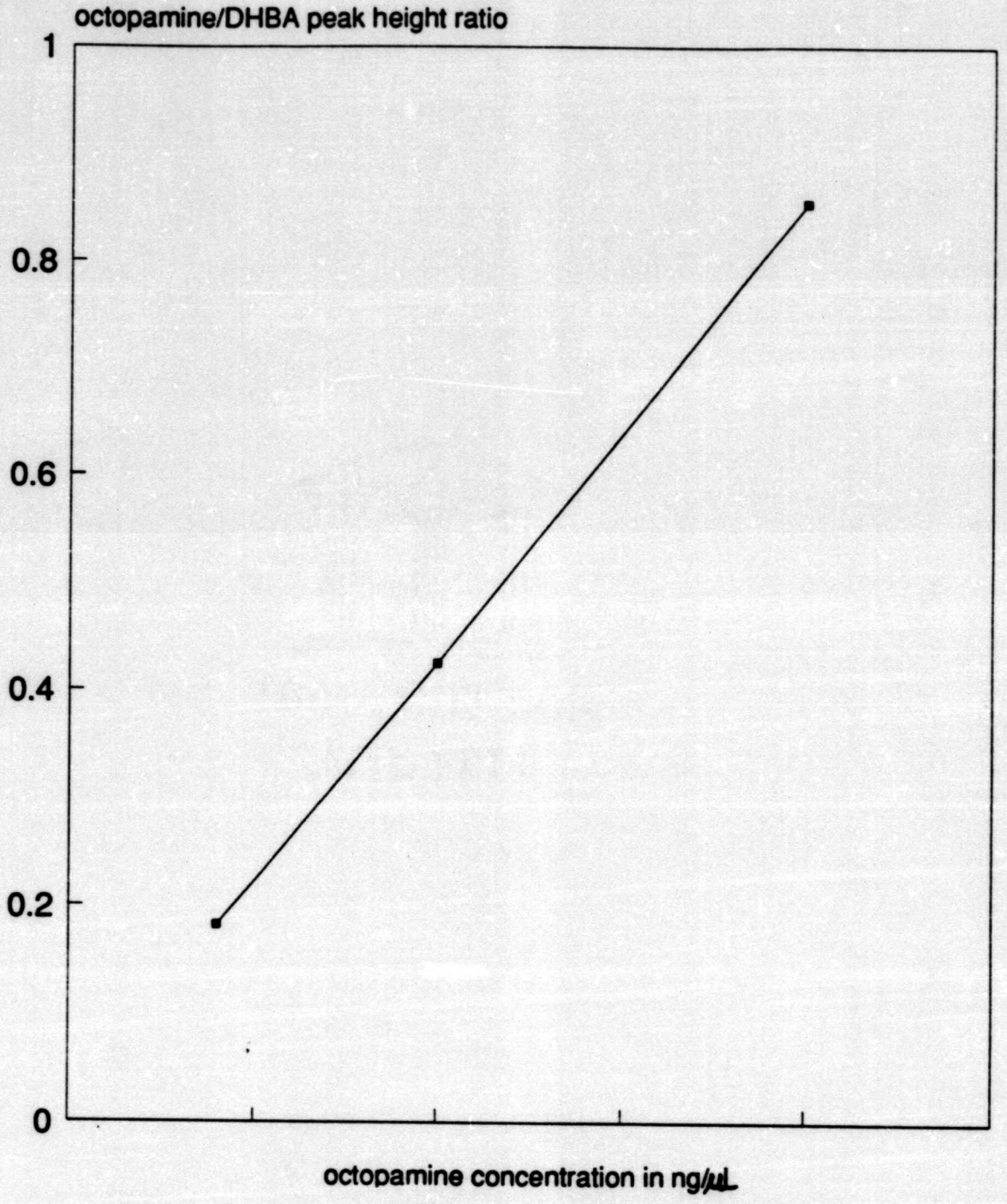


Figure 15. Standard curve for determining brain octopamine level.

$$SD_w = \sqrt{\frac{n_a(S_a^2 + M_a^2) + n_b(S_b^2 + M_b^2) + \ldots n_k(S_k^2 + M_k^2)}{n_a + n_b + \ldots + n_k} - M_T^2}$$

where $n_a \ldots n_k$ are the number of data points in each data set, $S_a \ldots S_k$ are the sample standard deviations for each data set, $M_a \ldots M_k$ are the mean for all data points in each group, and M_T is the weighted mean for all groups[54]. The data points were analyzed and if necessary a verification run was performed on any questionable result. Finally, the analysis of variance, ANOVA, was performed. The results of the statistical analysis are shown in Tables 3-7, and Figure 16.

TABLE 3

Data for the concentration of octopamine in the cockroach brain

REP # 1-5	Octopamine/ DHBA peak height ratio	Average ratio	[OCT]/ 2 br. ng	AVG [OCT] ng	[OCT] brain ng	AVG tot. ng
LN1	0.34		2.49		1.25	
	0.35		2.61		1.31	
	0.32	0.34	2.24	2.45	1.12	1.23
LN2	0.36		2.74		1.37	
	0.54		4.97		2.48	
	0.50	0.47	4.47	4.06	2.03	1.96
LN3	0.02		9.15		4.58	
	0.02		9.15		4.58	
	0.03	0.02	13.66	10.60	6.83	5.33
LN4	0.13		8.38		4.19	
	0.11		7.29		3.65	
	0.11	0.12	7.29	7.69	3.65	3.83
LN5	0.01		4.65		2.33	
	0.01		4.65		2.33	
	0.02	0.01	9.15	6.15	4.56	3.07
MD1	0.28		1.74		0.87	
	0.27		1.62		0.81	
	0.25	0.27	1.38	1.58	0.79	0.82
MD2	0.53		4.47		2.24	
	0.52		4.35		2.18	
	0.62	0.56	5.42	4.75	2.72	2.38
MD3	0.18		8.67		4.34	
	0.19		9.24		4.62	
	0.19	0.19	9.24	9.05	4.62	4.53
MD4	0.13		8.38		4.19	
	0.11		7.29		3.65	
	0.13	0.12	8.38	8.02	4.19	4.01
MD5	0.01		4.65		2.33	
	0.01		4.65		2.33	
	0.02	0.01	9.15	6.15	4.58	3.08

TABLE 3 continued...

LF1	0.15		6.65		3.33	
	0.15		6.65		3.33	
	0.15	0.15	6.65	6.65	3.33	3.33
LF2	0.63		5.53		2.78	
	0.60		5.21		2.61	
	0.66	0.66	5.85	5.87	2.93	2.77
LF3	0.18		8.67		4.34	
	0.17		8.09		4.05	
	0.20	0.18	9.82	8.86	4.91	4.43
LF4	0.16		10.01		5.01	
	0.15		9.47		4.74	
	0.18	0.16	11.10	10.19	5.55	5.10
LF5	0.02		9.15		4.58	
	0.02		9.15		4.58	
	0.02	0.02	9.15	9.15	4.58	4.58
MN1	0.16		7.13		3.56	
	0.16		7.13		3.56	
	0.15	0.16	6.65	6.97	3.33	3.49
MN2	0.60		5.21		2.61	
	0.55		4.68		2.34	
	0.51	0.55	4.26	4.72	2.13	2.36
MN3	0.14		6.36		3.18	
	0.17		8.09		4.05	
	0.15	0.15	6.94	7.13	3.47	3.56
MN4	0.19		11.65		5.83	
	0.19		11.65		5.83	
	0.20	0.19	12.19	11.80	6.10	5.92
MN5	0.15		6.65		3.33	
	0.15		6.65		3.33	
	0.13	0.14	5.70	6.18	2.85	3.09

LN = Lights on, MD = Midday, LF = Lights off, MN = Midnight, AVG pk. = average peak height, AVG tot. = average total, AVG = average, [OCT]/2 br.ng = octopamine concentration in nanograms/brain.

TABLE 4.

Results from the regression analysis for 5 REPS

REP #	df	B	M	R^2
1	1	0.139	16.144	0.995
2	1	0.106	18.936	0.974
3	1	0.029	3.477	0.999
4	1	-0.239	3.672	0.982
5	1	-0.000	0.444	0.990

REP # = replication number, df = degrees of freedom, B= y intercept, M= slope.

TABLE 5

Statistical results of the octopamine determination in the cockroach brain.

REP #	[Oct] ng/brain			Avg	wAvg	sSTD	wSTD
LN1	1.25	1.31	1.12	1.23	0.10		
LN2	1.37	2.48	2.03	1.96	0.56		
LN3	4.58	4.58	6.83	5.33	1.30		
LN4	4.19	3.65	3.65	3.83	0.31		
LN5	2.33	2.33	4.56	3.07	3.08	1.29	1.68
MD1	2.38	3.56	2.14	2.69	0.76		
MD2	2.24	2.18	2.72	2.38	0.30		
MD3	4.34	4.62	4.62	4.53	0.16		
MD4	4.19	3.65	4.19	4.01	0.31		
MD5	2.33	2.33	4.58	3.08	3.34	1.30	1.07
LF1	3.33	3.33	3.33	3.33	0.00		
LF2	2.78	2.93	2.61	2.77	0.16		
LF3	4.34	4.05	4.91	4.43	0.44		
LF4	5.01	4.74	5.55	5.10	0.41		
LF5	4.58	4.58	4.58	4.58	4.04	0.00	0.90
MN1	3.56	3.56	3.33	3.48	0.13		
MN2	2.61	2.34	2.13	2.36	0.24		
MN3	3.18	4.02	3.47	3.56	0.43		
MN4	5.83	5.83	6.10	5.92	0.16		
MN5	3.33	3.33	2.85	3.17	3.70	0.28	1.22

LN = Lights on, MD = Midday, LF = Lights off, MN = Midnight, Avg = average, wAvg = weighted average, sSTD = sample standard deviation, wSTD = weighted standard deviation, [OCT] = octopamine concentration, ng = nanogram.

TABLE 6

Comparison of results for REPS 1-5 from octopamine analysis in the cockroach brain

REP#	[Oct] ng/brain				Avg	wAvg	sSTD	wSTD
LN1	1.25	1.31	1.12	1.23			0.10	
MD1	2.38	3.56	2.14	2.69			0.76	
LF1	3.33	3.33	3.33	3.33			0.00	
MN1	3.56	3.56	3.33	3.48		2.68	0.13	0.97
LN2	1.37	2.48	2.03	1.96			0.56	
MD2	2.24	2.18	2.72	2.38			0.30	
LF2	2.78	2.93	2.61	2.77			0.16	
MN2	2.61	2.34	2.13	2.36		2.37	0.24	0.45
LN3	4.58	4.58	6.83	5.33			1.30	
MD3	4.34	4.62	4.62	4.53			0.16	
LF3	4.34	4.05	4.91	4.43			0.44	
MN3	3.18	4.02	3.47	3.56		4.46	0.43	0.96
LN4	4.19	3.65	3.65	3.83			0.31	
MD4	4.19	3.65	4.19	4.01			0.31	
LF4	5.01	4.74	5.55	5.10			0.41	
MN4	5.83	5.83	6.10	5.92		4.72	0.16	0.90
LN5	2.33	2.33	4.56	3.07			1.29	
MD5	2.33	2.33	4.58	3.08			1.30	
LF5	4.58	4.58	4.58	4.58			0.00	
MN5	3.33	3.33	2.85	3.17		3.48	0.28	1.12

LN = Lights on, MD = Midday, LF = Lights off, MN = Midnight, Avg = average, wAvg = weighted average, sSTD = sample standard deviation, wSTD = weighted standard deviation, [OCT] = octopamine concentration, ng = nanogram.

TABLE 7

ANOVA results for brain octopamine levels in *L. maderae*

Source of variation		df	SS	MS	F = 2.46
$\bar{Y} - \bar{\bar{Y}}$	between groups	3	2.55	0.85	-----
$\bar{Y} - \bar{\bar{Y}}$	within groups	16	26.84	1.68	-----
					0.51

df= degrees of freedom, SS = sums of squares, MS = mean of squares, F = between group variance / within group variance.

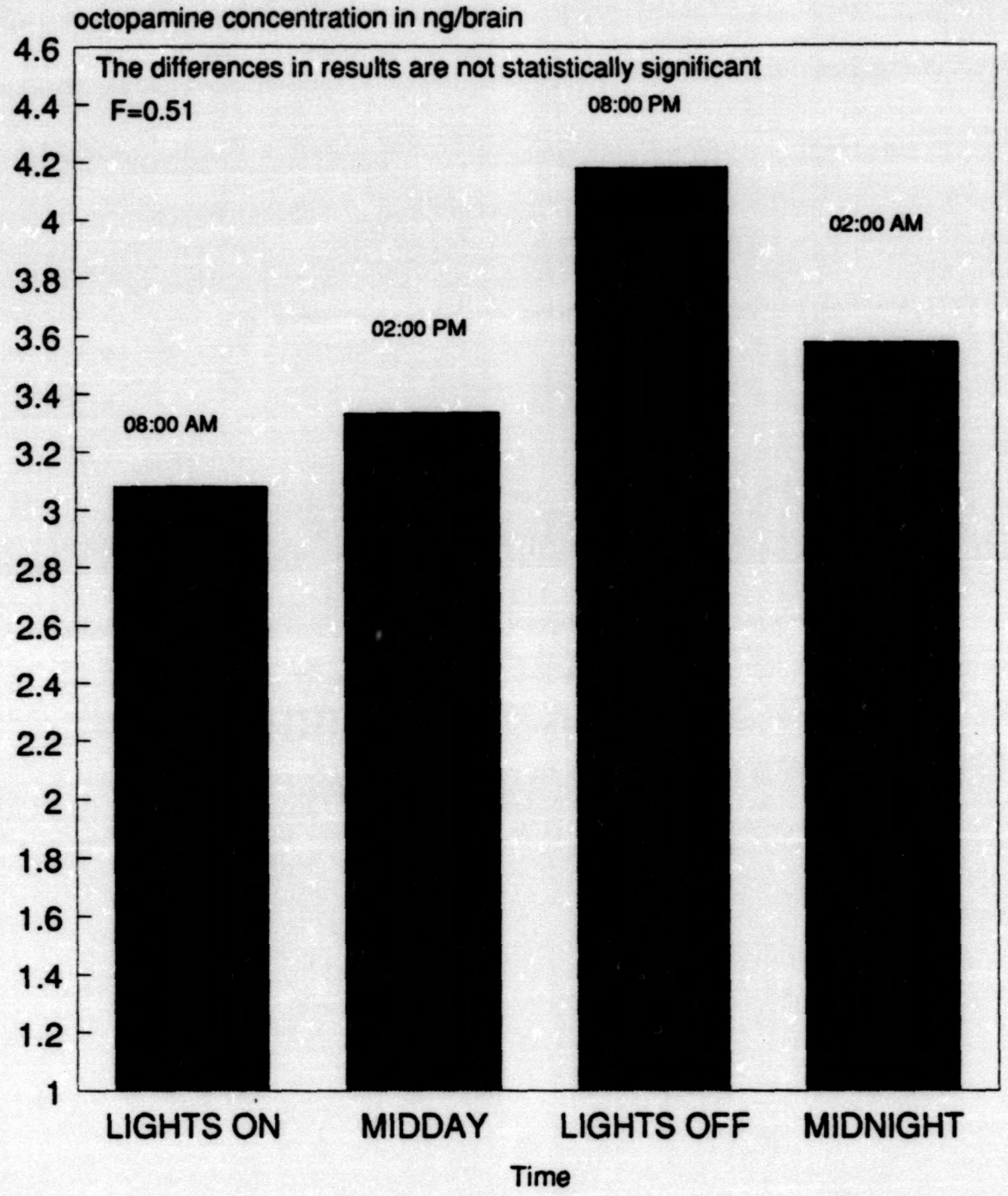
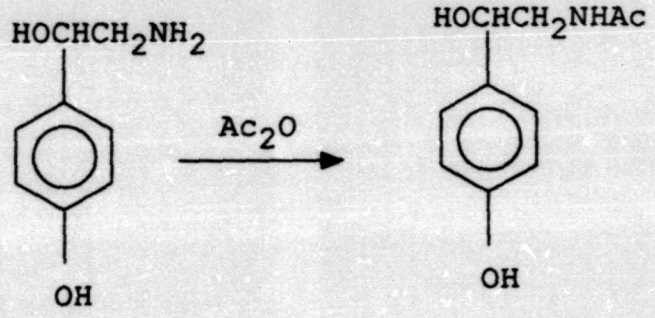
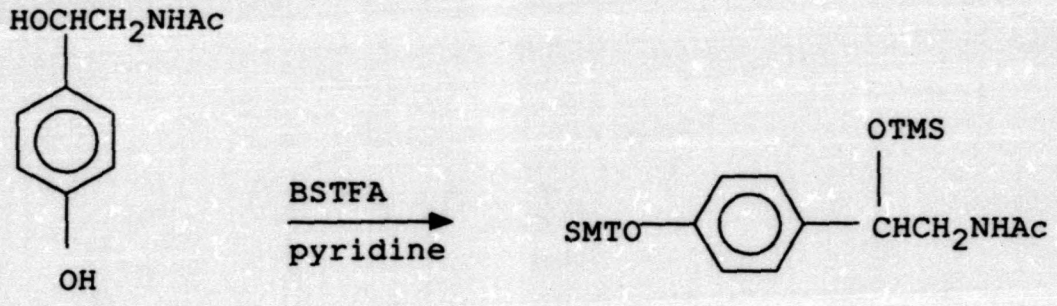


FIGURE 16. Variation of octopamine level per 24 hour period in the cockroach brain.

5. *Confirmation of octopamine by GC-MS.* For the GC/MS confirmation, the brain sample was treated the same as it was for HPLC analysis. The elution time of octopamine on the day of the fraction collection was determined to be 3 min and 45 sec. The total time of the peak was about 30 sec. The initial waiting time was determined by starting the stop watch at the point of injection, and timing until the first appearance of the peak. The peak width was determined by measuring the time in seconds it took from the beginning of the peak to the end of the peak. Upon establishing the appropriate collection time, the detector was turned off, and the column line disconnected at the detector so that the fraction was manually collected into a clean glass tube. The injection was repeated, and collection of fractions continued until an entire sample of brain extract from two cockroaches was used up. The fraction was then placed into a box of dry ice and transported along with a sample of pure unreconstituted standard of octopamine to Dr. Jan St. Pyrek's Mass Spectrometry Laboratory at the College of Pharmacy, University of Kentucky, Lexington, USA. In the mass spectrometry laboratory, the sample was transferred to the freezer until the analysis time. Pure octopamine standard was used to determine the optimum analysis parameters. The octopamine standard was dissolved into methanol, cooled on dry ice, then an equal volume of acetic anhydride was added,



octopamine N-acetylated octopamine



N-acetylated octopamine

O-silylated, N-acetylated octopamine

At the end of 30 min, enough HPLC grade water was added to dissolve excess acetic anhydride. This was followed by addition of a sufficent amount of ethyl acetate to allow the extraction of acetylated octopamine into the top ethyl acetate layer. The extraction was repeated one more time. The derivatized octopamine in ethyl acetate solvent was allowed to evaporate to dryness under a nitrogen atmosphere. At this time 50 µL of BSTFA derivatizing reagent was added. The GC/MS injector (split mode) / detector/ and the transfer line temperature were set to 280° C. The mass spectrometer electronics were set to scan from

detector/ and the transfer line temperature were set to 280° C. The mass spectrometer electronics were set to scan from 750 - 50 amu @ 0.3sec/decade, (750 -75 is 1 decade), with 0.3 seconds interscan. Electron impact was set at 70 eV. Characteristic peaks for the standard octopamine were at *m/z* 324, *m/z* 267, and *m/z* 73. The fraction from the cockroach brain extract was brought to room temperature, evaporated to dryness under the nitrogen atmosphere and then treated in the same manner as described for the octopamine standard. GC/MS results are shown in Figures 17-22.

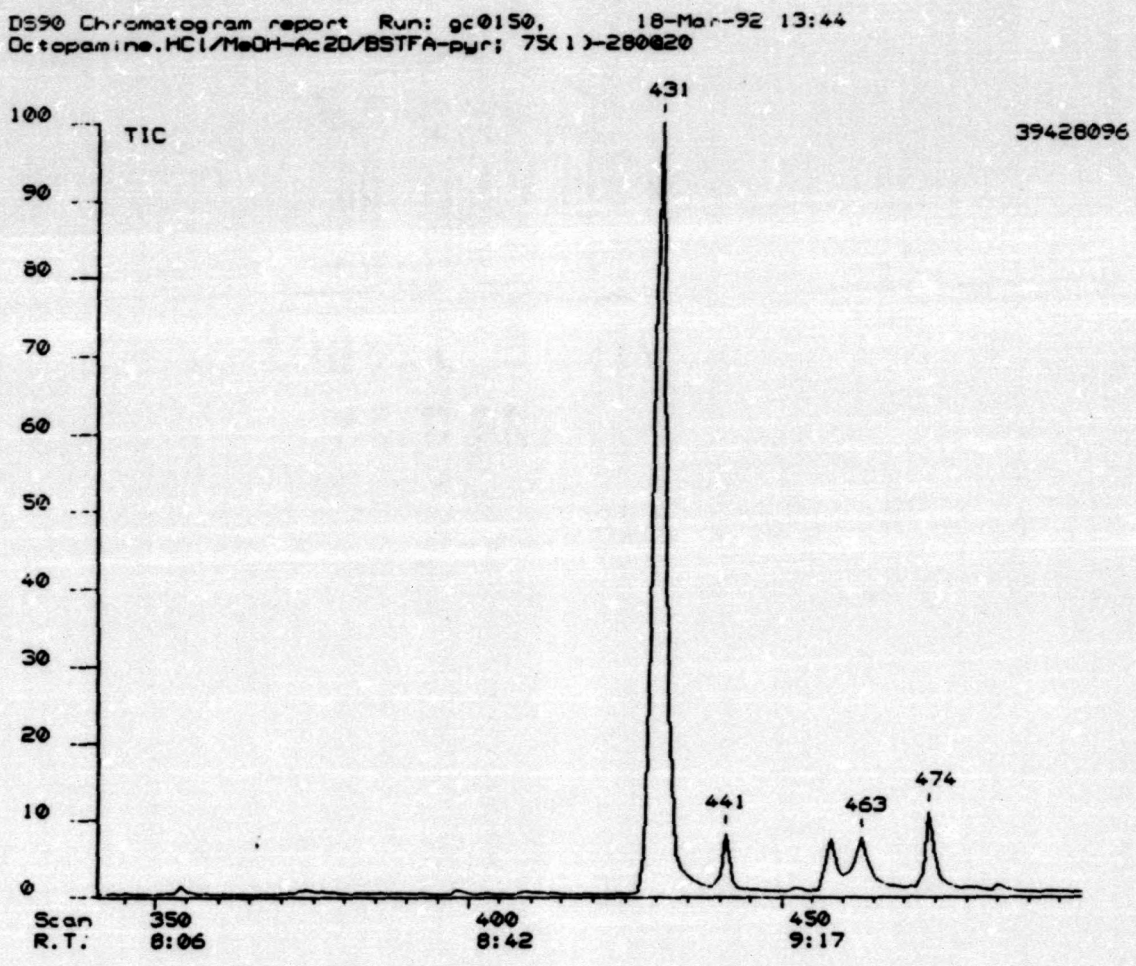


FIGURE 17. GC chromatogram of octopamine standard.

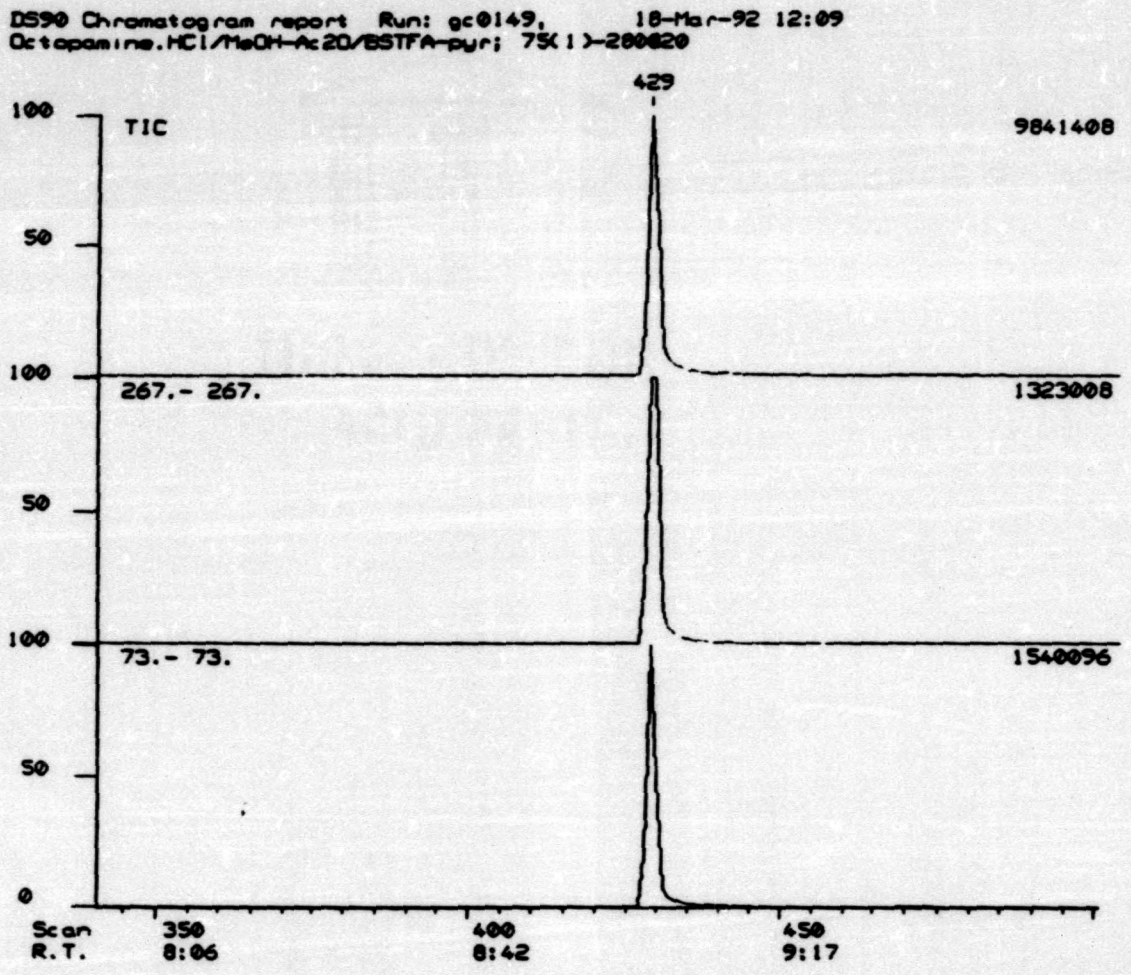


FIGURE 18. Ion chromatogram of octopamine standard.

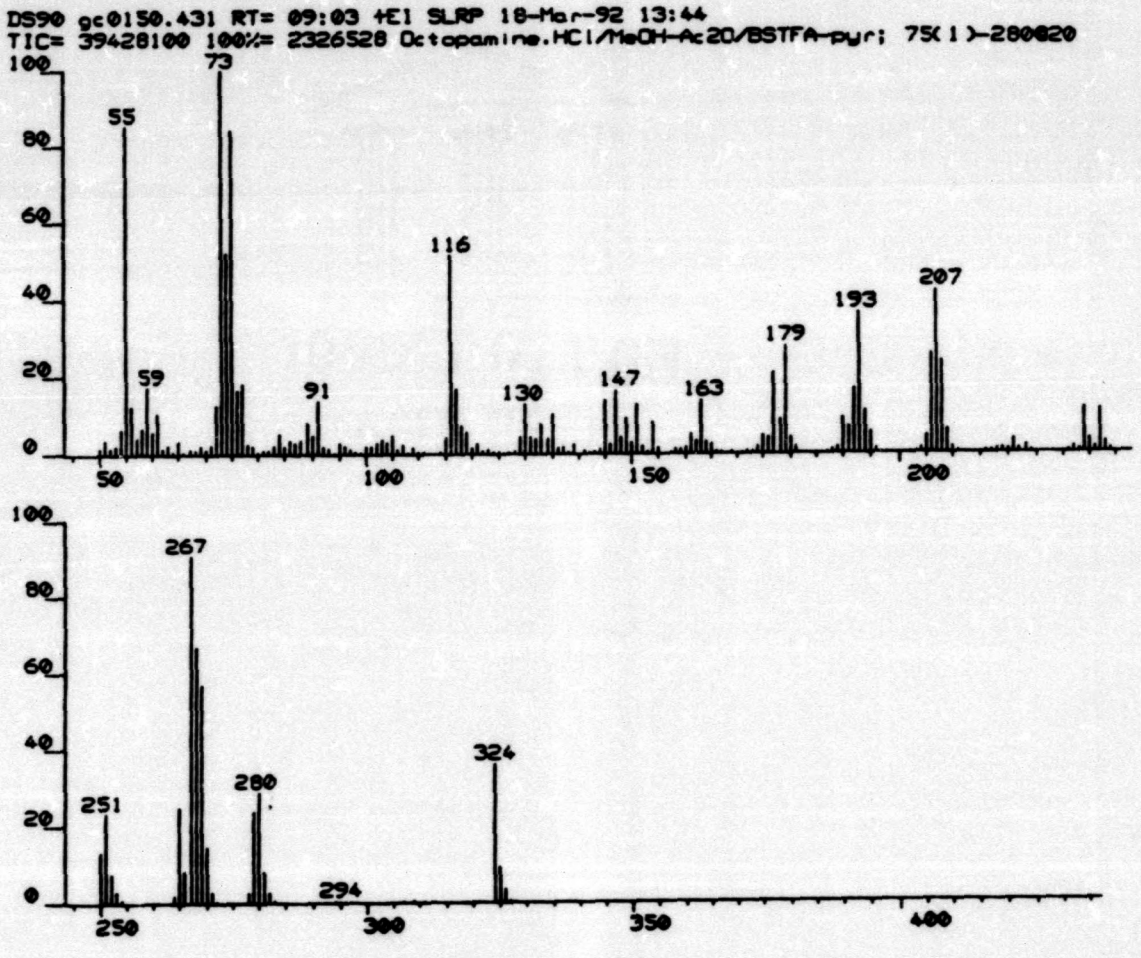


FIGURE 19. Mass spectrum of octopamine standard.

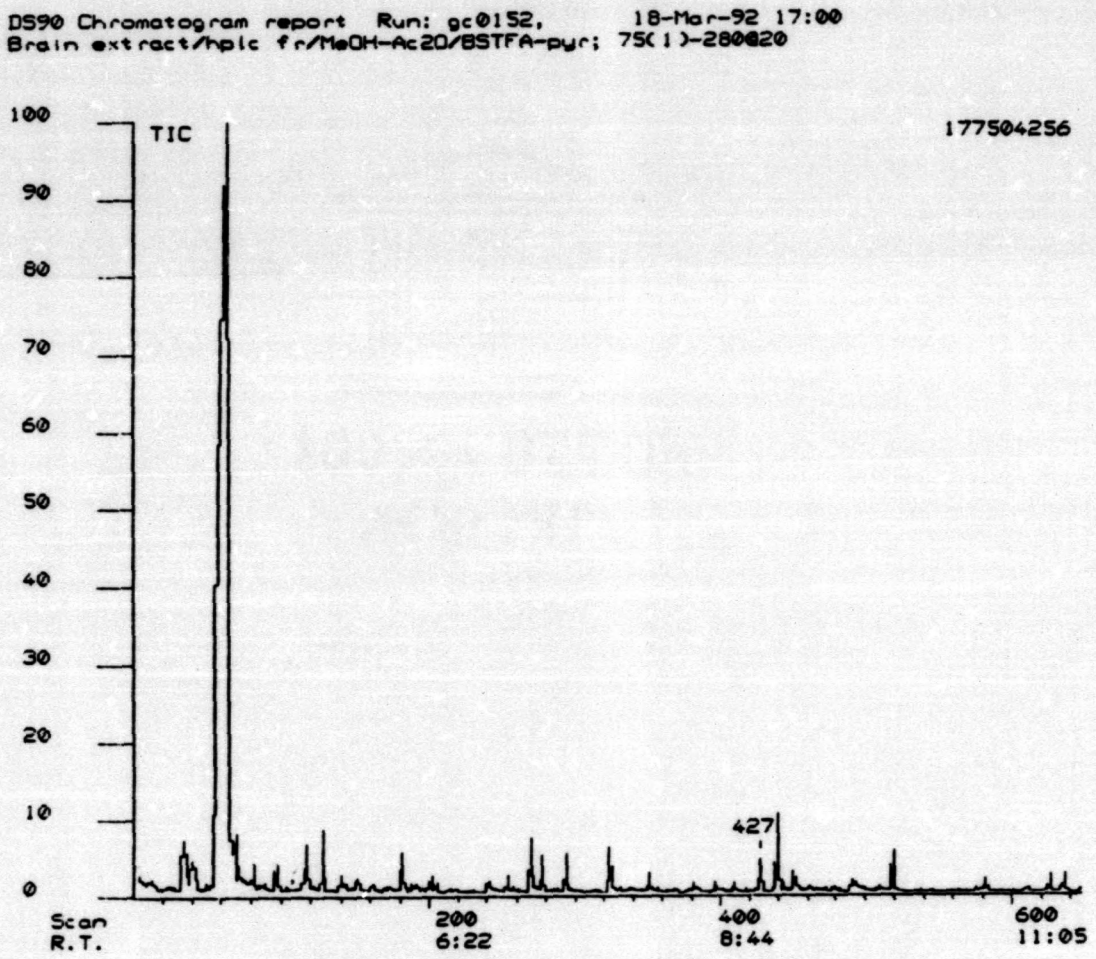


FIGURE 20. GC chromatogram of HPLC fraction of the cockroach *L. maderae* brain extract.

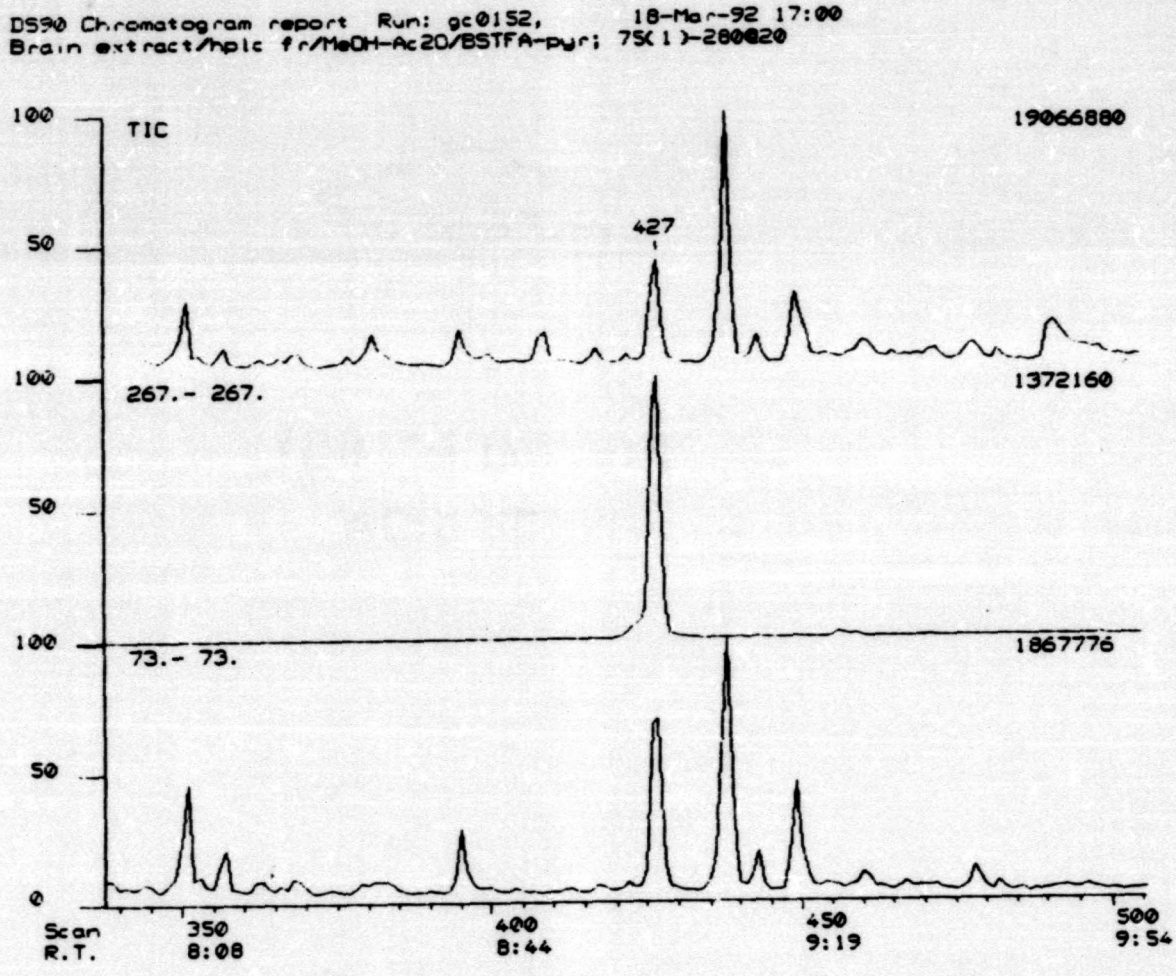


FIGURE 21. Ion chromatogram of the HPLC fraction of the cockroach *L. maderae* brain extract.

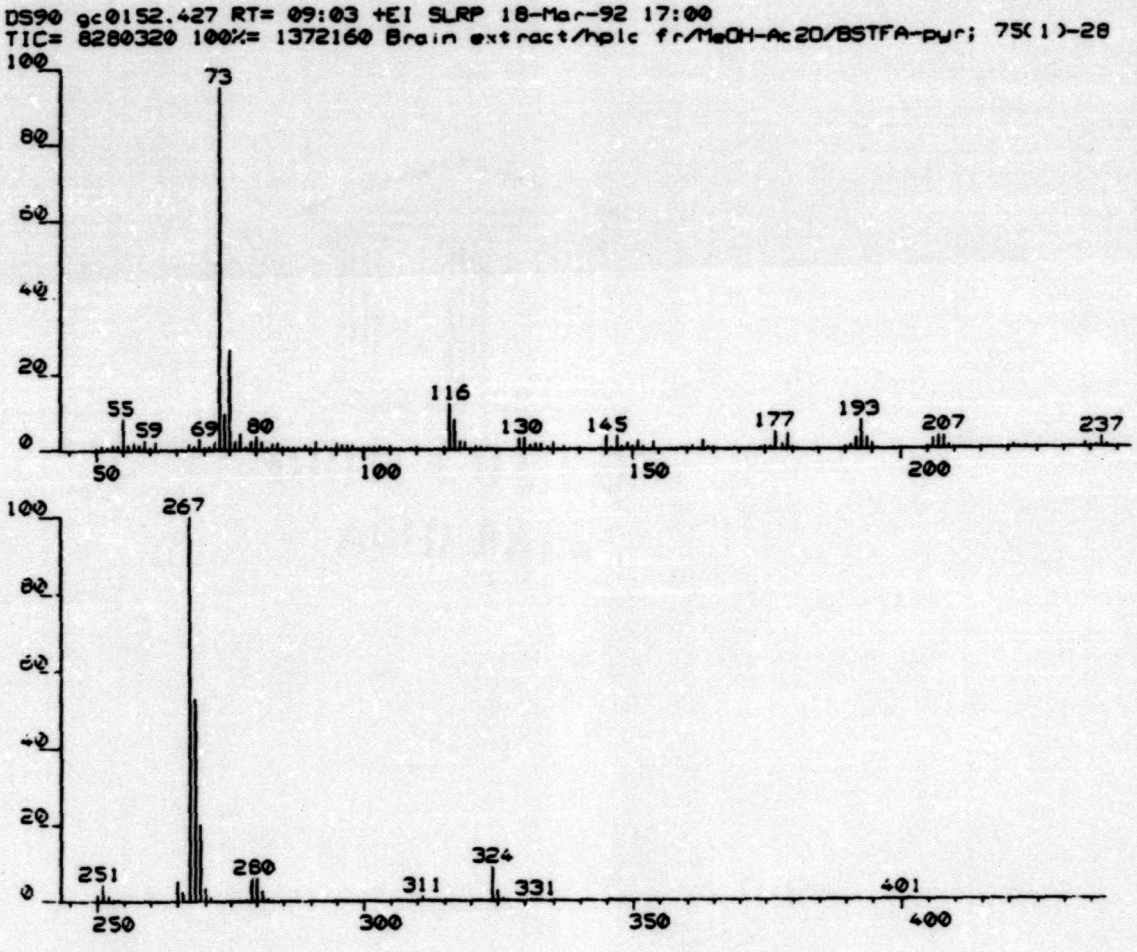


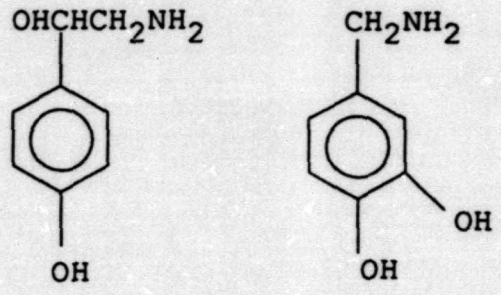
FIGURE 22. Mass spectrum of HPLC fraction of the brain extract from cockroach *L. maderae*.

CHAPTER III

DISCUSSION OF RESULTS

Initial study of octopamine shows that the octopamine is irreversibly oxidized as compared to our internal standard DHBA which is reversibly oxidized (Figure 2, 3). Literature does not offer information as to the possible reason why octopamine is irreversibly oxidized, except the fact that the reaction is irreversible. It is possible that the exchange of electrons at the electrode is too slow for the octopamine to be a reversible process, which then suggests that the reaction is not governed by thermodynamics but by kinetics. The overall reaction is not Nernstian. Without further study of the reaction mechanism it is not possible to answer the question concretely. In our study the scan range was large enough to be able to detect both the cathodic and the anodic peak. We found only the cathodic peak for octopamine, indicating irreversibility of the reaction, whereas there were both the anodic and the cathodic peaks present for DHBA. The anodic and the cathodic peaks for DHBA are highly separated, suggesting a pseudoreversibility. The question one may pose is why is octopamine electrochemically different from DHBA? Structurally the two compounds are similar, but chemically

very different. The structure of octopamine and DHBA is shown below:



Octopamine 3,4-dihydroxybenzylamine

It is not easy to answer this question, without additional study of octopamine oxidation products. DHBA is a catecholamine, whereas the octopamine is a phenolic amine. The OH group on the C_1 of the ethanolamine functional group of octopamine makes this particular bond easy to break, whereas the OH group on the ring is resonance stabilized and is thus harder to disturb. For example, during the oxidation at the electrode, DHBA's two OH functional groups may loose one hydrogen each which is quickly regained during the reverse scan. Octopamine on the other hand may lose its ethanolamine functional group irreversibly, or through rearrangement becomes a different molecule altogether. A study of the oxidation products of octopamine would afford an answer to the elusive behavior of octopamine. In our study we were concerned with finding the most suitable operating potential for octopamine which would also be compatible with our internal standard DHBA, enabling us to

quantitate octopamine in the cockroach brain. Hydrodynamic voltammograms of the two compounds in question show that the optimum potential for DHBA is around 0.75 to 0.95 V vs Ag/AgCl reference electrode, which agrees with the value reported by BAS [55]. We determined that the optimum applied potential for octopamine to be 1.040 V vs Ag/AgCl, as can be seen in Figure 4.

Our study revealed that the preinjection column mobile phase oxidation was unnecessary; however, there are a few points to bear in mind when using this high sensitivity method. These are mentioned here. It is very important to prepare the mobile phase with the utmost care. This is to prevent the precipitation of salts in the mobile phase which tend to accumulate on the surface of the electrode causing drift, and sometimes an outright electronic jam of the detector. An effect of salt accumulation at the electrode surface, air bubble passing through the electrode, and electronic interference is shown in Figure 23. Degassing the mobile phase thorougly can not be over-emphasized to prevent air bubbles from adhering to the electrode surface also causing the baseline drift in the upward direction. Cleaning the column regularly will also eliminate particulates that get accumulated on the column during the run, and which tend to elute occasionally during the analysis adding to excess peaks.

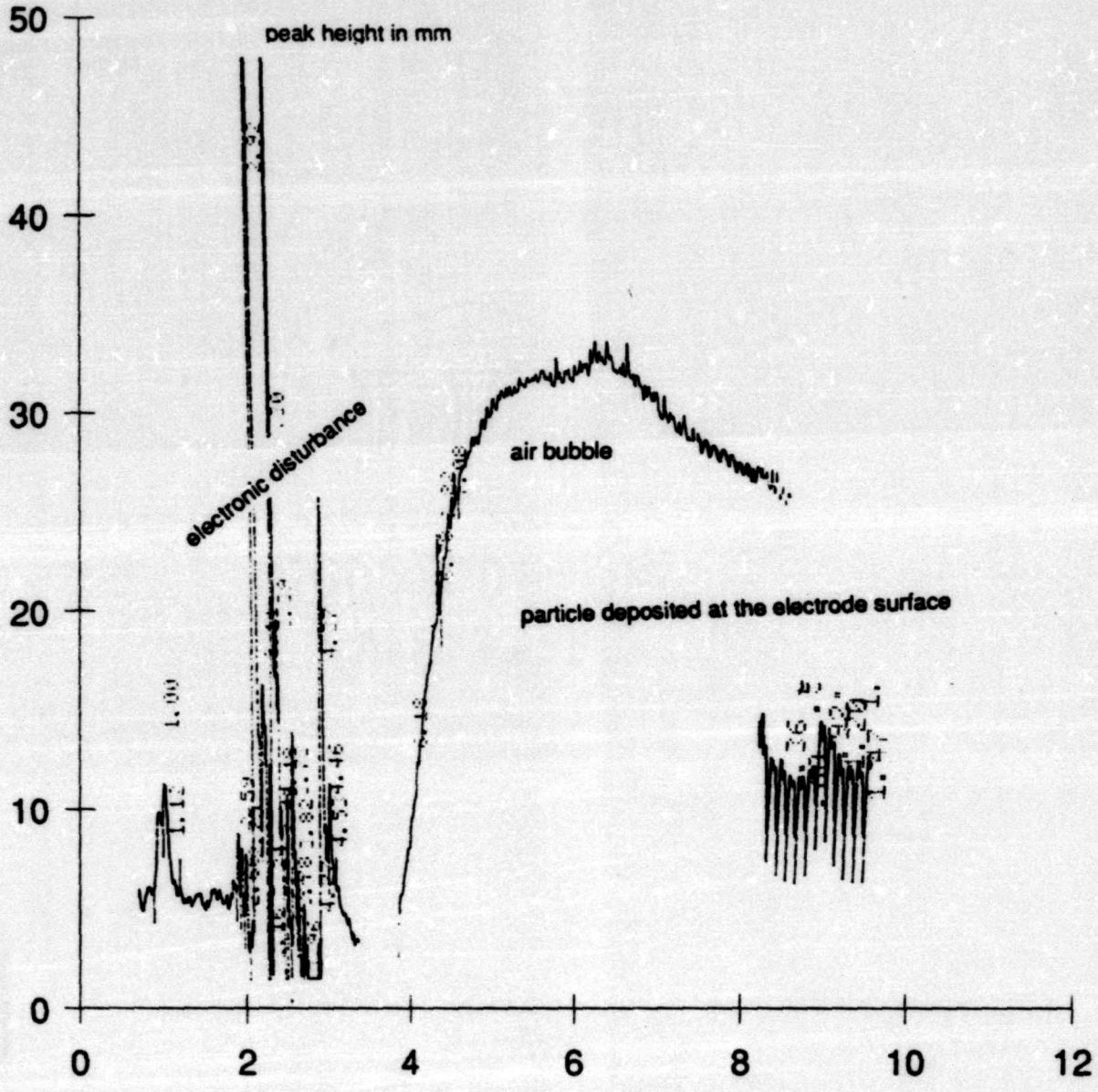


Figure 23. HPLC problems.

During our experiment we found that the best way to keep the column in good condition was to clean it for 10 h with 80 - 100 percent acetonitrile after the analysis of sample and prior to storage by letting the cleaning solvent run at the same elution volume as the sample was run. Prior to the next analysis, we allowed 80:60 methanol:HPLC grade water mixture to run for approximately 8-10 h before changing to the mobile phase. We found that this method gave a stable baseline in a relatively short time, 30 min to 3 h after the detector was turned on.

The limit of detection for our instrument was found to be around 70 pg of octopamine as seen in Figure 5.

The remarkable results obtained using solid phase extraction NH_2 column in conjuction with methylene chloride/methanol conditioning solvent is shown in Figure 8. The amino solid phase extraction column was chosen by assuming that there are many small molecular weight, highly polar, non- hydroxy amino molecules left in the initial brain extract that interfered with octopamine analysis on the HPLC C_{18} reverse phase column. The amino solid phase extraction column with our conditioning solvent afforded a significant retention of these interferring compounds and allowing only the desirable analyte to pass through for subsequent HPLC analysis. Our method simplified the sample matrix, making it less complex and easier to analyze. The major driving force behind the analyte isolation in solid phase extraction is a differential affinity for the analyte

between the solvent and the packing material. This means that the octopamine and DHBA in our study have greater affinity for the mobile phase than for the packing material, and are thus eluted almost 100 percent using one LC-NH_2 SPE column, and about 80 percent if using two columns in sequence and bypassing further washing of the sample from the second column, as was determined from the percent recovery study in Figure 10. The interferring substances are retained on the solid phase extraction column. The capacity of the solid phase extraction column is not unlimited. In our study we found that using 1 mL solid phase extraction column was not sufficient to clean up two brain samples satisfactory. We therefore used two 1 mL solid phase extraction columns in series. An alternative would have been going to a larger capacity solid phase extraction column. However, this option is not desirable due to the dilution factor involved using a larger volume solid phase extraction column. It must be pointed out that a 2 mL or larger column size would have been just as efficient as were the 1 mL solid phase extraction columns we used in our experiment. Our sample size required us to use the smaller volume solid phase extraction columns.

Our HPLC system utilized reverse-phase ion-pair chromatography with amperometric electrochemical detection. The terms reverse-phase ion-pair chromatography and amperometric detection need clarification. Ion-pair chromatography is essentially an alternative to ion-exchange

chromatography for the separation of ionic compounds. In order to make the ionic compound of interest interact with the nonpolar stationary phase a counter ion is added to the mobile phase. The ionic compound of interest and the counter-ion form an ion pair that when combined have sufficient lipophilic character to be retained by the nonpolar column. If the counter-ion was not present, the compound of interest would not be retained by the column at all, or it would exhibit severe tailing. In our experiment, the octopamine and DHBA were the two ionic compounds of interest, and the ion-pair reagent we used was 1-heptane sulfonic acid sodium salt. The ion-pair and our analyte probably formed the coupled neutral species according to the following equation:

$$X^+_{aq} + Q^-_{aq} \Longleftrightarrow [X^+Q^-]_{aq} \Longleftrightarrow [X^+Q^-]_{org}$$

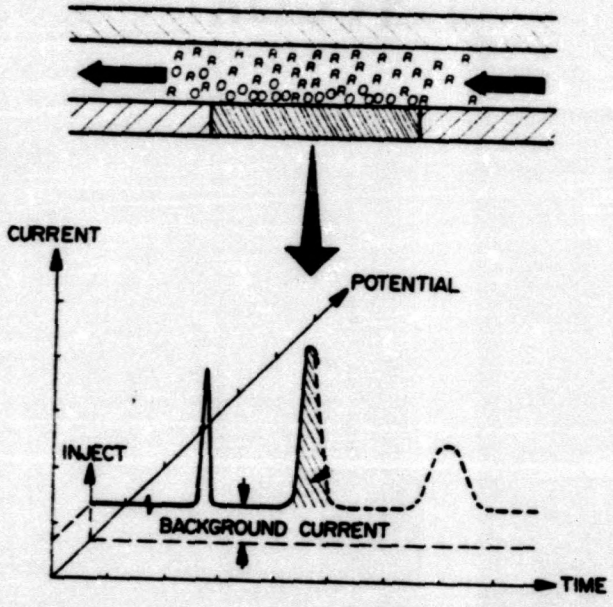
where X^+ is the protonated amino group on the octopamine and DHBA, and Q^- is the deprotonated sodium salt of 1-heptanesulfonic acid with the negative charge being on one of the oxygens. In this form the coupled ion-pair is retained on the column long enough to allow a good separation of the octopamine and DHBA from each other and other eluting species. The amount of counter-ion added to the mobile phase is the major factor governing retention time of the analyte. Increasing the amount of counter-ion will increase the retention time of the analyte within

limits. Also the pH of the mobile phase is obviously important in the formation of an ion-pair. The pH of the mobile phase if not chosen carefully, can cause a variety of problems, for example, at higher pH (ie. >7.0), there appeared to be considerable precipitation of the components in the mobile phase, which contributed to a lengthy delay in the progress of our experiment. Temperature also has an effect on the eluting compounds. At higher temperatures, the elution is faster, thereby shortening the retention time.

Reverse-phase chromatography is simply the reverse of normal-phase chromatography. The mechanism is based on the immiscibility of the stationary phase with the mobile phase. In reverse-phase the stationary phase on the column is nonpolar, for example having an C_8 or C_{18} functional group. The separation in reverse-phase chromatography is governed by the partitioning of the analyte between the stationary and mobile phase. The more soluble the analyte is in mobile phase, the faster it will elute. To manipulate the solubility of the analyte in the mobile phase, organic modifiers are normally added. The most commonly used organic modifier added to the mobile phase is methanol or acetonitrile. In our experiment, we found that 6% methanol added to the mobile phase gave good resolution and retention time.

Amperometric electrochemical detection is a very sensitive and selective analytical technique. It is used

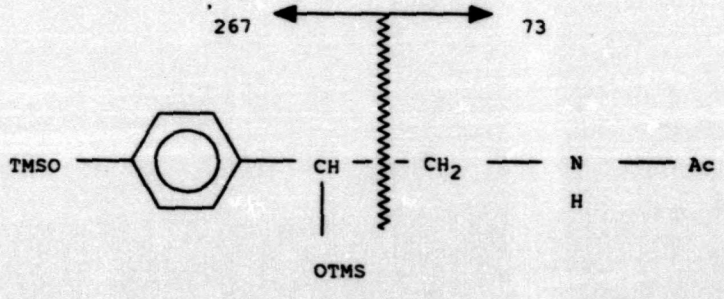
for the detection of electroactive substances, and has found widespread use in neurochemistry. It is a surface technique, and a small volume surface cell is required. In our experiment, the thin layer cell consisted of a glassy carbon electrode with a cell volume of less than 5 μL volume. As an electrochemically active compound passes over an electrode held at a potential sufficiently great for an electron transfer to occur, a current is produced which is directly proportional to the concentration of the compound entering the electrochemical cell. As the concentration changes as a function of time due to elution of the compound from the chromatographic column, so does the current. This is the basis for quantitative electrochemical analysis. The schematic representation of the amperometric detection is shown below:

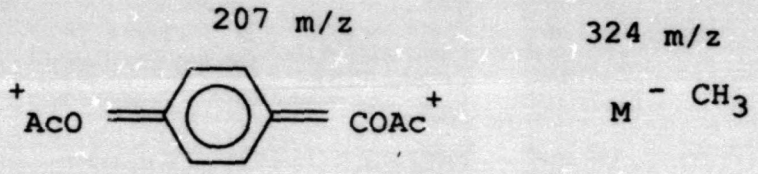


Column resolution, and adjustment of the electrode potential improves the selectivity of amperometric detection. In our experiment it was determined from Figure 4, that the optimum applied potential for octopamine analysis is 1.040V vs Ag/AgCl reference electrode.

The methodology for sample clean-up using LC- NH_2 solid phase extraction columns afforded an excellent recovery. We did not encounter the problems with the amperometric detection as reported by Bailey et al.[22] as discussed in the introduction and as can be seen in Figures 8, and 12-13. The problems we experienced were operator related, wear and tear of consumable instrumental components and due to improperly prepared mobile phase. These effects are shown in Figure 23.

Octopamine when subjected to elevated temperatures is subject to fragmentation even when partially derivatized. Figures 13 - 18 show the results of octopamine confirmation by EI GC/MS. The two most abundant ions present are the 267 *m/z* and 73 *m/z*, and two ions of lesser abundance of 324 *m/z* and 207 *m/z*. The origin and structures of these ions are shown below:





Comparing Figure 19 and Figure 22, it was found that the mass spectrum of brain octopamine exhibited the ions of the same mass to charge ratio as the standard octopamine confirming that the compound in our analysis was indeed octopamine.

Examining Figure 16, it at first appears that the octopamine exhibits a daily rhythm. But upon analysis of variance, ANOVA, it was concluded that the F ratio was not statistically significant, thereby indicating that the octopamine does not exhibit a daily rhythm as postulated.

CHAPTER IV

SUMMARY

In conjuction with our study to determine the existence of a daily rhythm for brain octopamine in the cockroach *L. maderae*, we developed a convenient, inexpensive, and very sensitive method for the determination of octopamine. The procedure involved utilizing NH_2 solid phase extraction columns to clean up the sample prior to HPLC reverse-phase ion-pair with amperometric electrochemical detection. Our methodology affords excellent recovery of octopamine from the solid phase extraction, and offers the detection of this compound in picogram levels. The presence of octopamine in the cockroach brain extract was confirmed by electron impact GC/MS. This study found that the octopamine levels in cockroach brains do not exhibit a daily rhythm.

Based on the reports in the literature regarding the function of octopamine in the insects, as discussed in the introduction, it would be of interest to further investigate the levels of octopamine found in the hemolymph of the cockroach. It is very possible that a daily rhythm would be found.

CHAPTER V

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