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Utilization of a derivatization method with multidimensional liquid chromatography-mass spectrometry for the detection of neurotransmitters

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Thesis

**UTILIZATION OF A DERIVATIZATION METHOD WITH
MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY-MASS
SPECTROMETRY FOR THE DETECTION OF NEUROTRANSMITTERS**

by

OLIVIA KAYE GEORGE

B.S., Mercer University, 2018

Submitted in partial fulfillment of the
requirements for the degree of
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Approved by

First Reader

Sabra Botch-Jones, M.S., M.A., D-ABFT-FT
Assistant Professor, Program in Biomedical Forensic Sciences
Department of Anatomy & Neurobiology

Second Reader

Claude Mallet, Ph.D.
Senior Consulting Scientist
Waters Corporation

Third Reader

Joshua Wilhide, M.S.
MCAC Manager
University of Maryland Baltimore County

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ABSTRACT

Neurotransmitters are endogenous compounds located in the brain and can occur in other parts of the body at low concentrations. They are a challenging group of compounds when it comes to analysis. Compounds with amine functionality such as dopamine and serotonin are sensitive to high pH and light and can spontaneously oxidize and degrade. Since neurotransmitters exist at low levels, it can be difficult to achieve sufficient spectrometry data and clean chromatography. In order to improve data acquisition, derivatization and the use of multidimensional liquid chromatography (2D-LC) was evaluated. Dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), serotonin, tyrosine, tryptophan, and noradrenaline were the compounds of interest with tyramine included as an internal standard. Data was collected before and after derivatization to compare the resulting chromatography. Chromatography was completed using a 6x6 grid of methods with variables of organic solvent, elution pH, loading pH, and trapping column chemistry utilizing different elution column chemistries. Derivatization was examined with Dabsyl-Cl and Dansyl-Cl at pH 8.5, 9.5, 10.5, and 14. A final method was chosen with Dabsyl at pH 8.5 for the Multiple Reaction Monitoring (MRM) scans. Before derivatization, the chromatography had poor peak shapes such as tailing, fronting, or shouldering or too much

distortion to be able to distinguish a peak for all of the examined methods. Resulting chromatography after derivatization showed overall improvement in peak shape and intensity for a majority of the methods. Derivatization aided in increasing the mass and stability of the compounds which allowed for more sensitive detection. Multidimensional liquid chromatography improved the separation of structurally similar compounds and increased sensitivity. By combining the two, better analysis of neurotransmitters was possible.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADHD	Attention Deficit Hyperactive Disorder
amu	Atomic Mass Unit
BSM	Binary Solvent Manager
°C	Degrees Celsius
DAB	Dabsyl-Cl
DAN	Dansyl-Cl
EA	Ethyl Acetate
FA	Formic Acid
HLB	Hydrophilic Lipophilic Balance
HPLC	High Performance Liquid Chromatography
kV	Kilovolts
L/hr	Liters per hour
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
L-DOPA	L-3,4-dihydroxyphenylalanine
m/z	Mass to charge ratio
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter

MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
ng	Nanogram
pKa	Acid dissociation constant
PMNT	Plasma Membrane Neurotransmitter Transporter
pmol	Picomoles
ppb	Parts per billion
ppm	Parts per million
QSM	Quaternary Solvent Manager
Qual	Qualitative
Quant	Quantitative
SIR	Selected Ion Recording
SPE	Solid Phase Extraction
UPLC	Ultra-Performance Liquid Chromatography
USA	United States of America
V	Volts
VNT	Vesicular Neurotransmitter Transporter
µg	Microgram
µL	Microliter
µm	Micrometer

INTRODUCTION

Neurotransmitters

Neurotransmitters and their effects have been a popular point of study in a variety of fields from neuroscience and psychology to forensics and pharmacology [1]. Their prevalence in recent scientific literature has increased in parallel with the understanding of the brain. The best approach to starting analysis is to understand their function and roles inside of the body. Neurotransmitters are endogenous compounds located in the brain and can occur in other parts of the body at low concentrations [1]. The release of neurotransmitters is facilitated by electrochemical pathways that signal neurons to fire. The pre-synaptic neuron forms an envelope around the neurotransmitter called a vesicular neurotransmitter transporter (VNT). The VNT binds to the outside edge of the neuron and releases the neurotransmitter into the synaptic cleft. The neurotransmitter is received by the plasma membrane neurotransmitter transporter (PMNT). Each PMNT is designed to fit a specific neurotransmitter based on its structure [3]. Once received, the neurotransmitter is released into the post-synaptic neuron [2, 3]. The transmission of the compounds is vital for aiding specific brain functions whether related to hormones or peripheral body functions [4, 5]. Dopamine, for instance, is related to motivation, cardiovascular activities, motor function, and the balance of mood. Serotonin levels are connected to sleep cycles, sensory perception, mood, appetite, and sexual motivation [4-6]. Much like the affinity of the PMNTs, neurotransmitters are classified by their structure. Classifications include amino acids such as glutamate and GABA, amines or

catecholamines like norepinephrine and dopamine, and peptides such as oxytocin and vasopressin [1, 5]. The experiment discussed here focuses on the analysis of amines: dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), serotonin, noradrenaline, tyrosine, and tryptophan with tyramine as an internal standard. Each compound has either a primary or secondary amine functional group attached on the carbon chain connected to an aromatic structure (Figure 1).

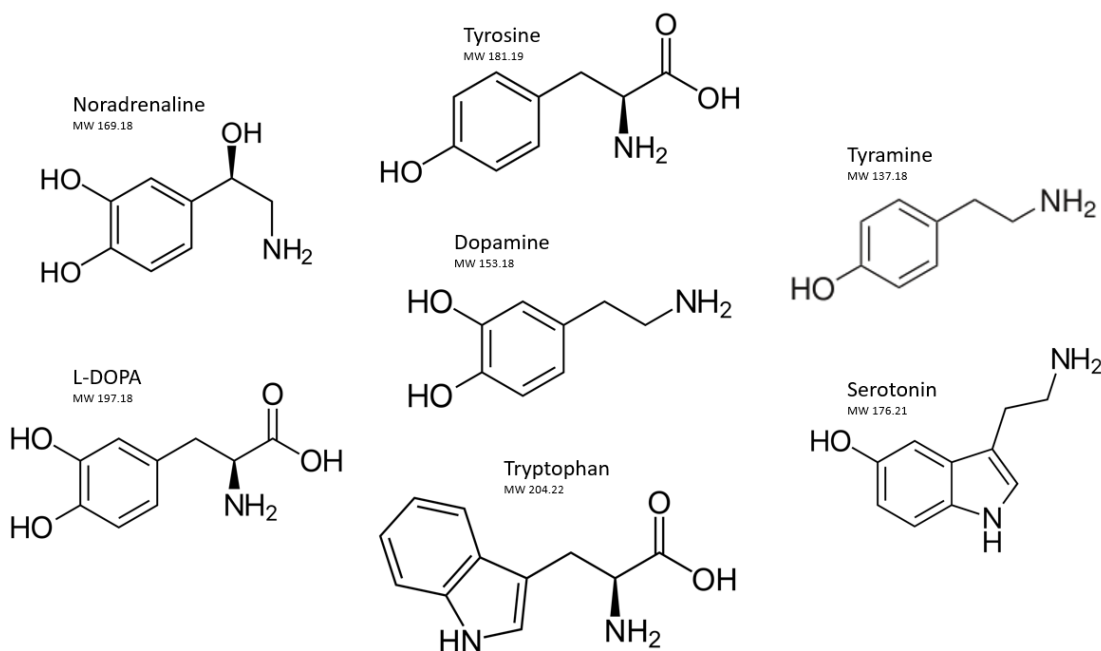


Figure 1. Chemical structure of analyzed neurotransmitters [37-43].

Regulation of neurotransmitters within the body is important for maintaining a state of homeostasis and ensuring that all functions are normal. Dysfunction in the reuptake or metabolism of neurotransmitters can either cause the brain to produce too much or too little. Inhibitors prevent the reuptake and will signal as if there is nothing present, therefore causing an overproduction of a specific neurotransmitter [3]. Additional abnormalities in the synaptic cleft and use of drugs of abuse can also cause overproduction as well as

underproduction. [3, 7] The dysregulation can lead to a wide range of physical and mental disorders such as depression, schizophrenia, Tourette's Syndrome, bipolar disorder, attention deficit hyperactive disorder (ADHD), Parkinson's, addiction, and hypertension [8-12]. Some studies show that higher serotonin levels are also seen in patients with metastatic carcinoid tumors [13].

Forensic Relevance

As mentioned above, drugs of abuse can have a significant impact on the concentration of neurotransmitters in the brain. They can extend the release especially if their structures are similar to endogenous compounds. The similar structure allows for less secure bonding on the PMNTs and disruption of the reuptake of the neurotransmitter for which it was designated [1]. Prescription drugs for neurological disorders also have an influence on regulation. In most cases the prescribed medication is responsible for facilitating the increase of neurotransmitter production. However, some exist to decrease the production such as in cases of ADHD and schizophrenia [22]. Both prescribed and illicit drugs can be detected in cases of drug abuse, emergency toxicological screenings, and forensic medical examinations [2]. Other examinations will measure the neurotransmitter levels directly, but complications occur when attempting to decipher whether the source of the neurotransmitter is endogenous or from an external source. Some studies have examined the connection of neurotransmitters to levels of aggression especially in criminal cases due to the influence on reward mechanisms, risk taking, and mood. Consideration of neurotransmitter levels have been taken into account in forensic

examinations for pursuing an insanity plea. While mental disorders and dysfunction can be related to the commission of crimes, other external factors such as childhood trauma, family dynamics, and peer relations also have a prominent influence [14].

Challenges with Analysis

Analysis of neurotransmitters can be quite challenging due to their inherent chemical and physical properties. Several compounds such as dopamine and noradrenaline have sensitivity to light and high pH which can cause degradation [1]. Both compounds also undergo spontaneous oxidation under certain conditions and have multiple pKa values that gives them zwitter ionic behavior. [1]. Most neurotransmitters will occur at low levels which requires lower detection limits. The limitation is partially due to metabolism degradation as well as matrix effects and ion suppression [4]. Due to the presence of endogenous levels, analysis can be difficult when trying to determine whether or not the presence is from an outside source [2]. Current analytical methods are lacking in levels of detection which is an issue when examining a compound at levels lower than 0.57 pmol/mL [22] and means to compensate for the zwitter ionic behavior. Some compounds also have similar structures to each other and will elute at similar times. To compensate, scientists have developed methods with lengthy run times and intensive sample preparation hoping to improve separation. Additionally, they use reversed phase separation which has difficulty when the compounds have multiple pKa values. [5, 10, 15]. The issue can be partially addressed with the use of a derivatization process.

Derivatization

One approach for the analytical challenges mentioned is through the use of derivatization. Derivatization is the alteration of the chemical structure of a compound to increase its utility in analysis. The transformation into a derivative product is dependent on the selection of a derivatization agent [16, 17]. An agent is chosen based on its structure and its ability to bind to specific functional groups. Binding can provide additional means of detection such as fluorescence, isotopic tagging, or increased tandem mass spectrometry (MS/MS) fragmentation signatures. Derivatization can increase stability and improve chromatography to aid in identification and quantitation. The improvement can be seen in signal enhancement as well as increased separation [16-18]. In the following experiments, considerations were given to derivatization agents that were able to bond to amine or phenol functionality. Studies on amines have examined the use of Dansyl-Cl [18-22], Dabsyl-Cl [18, 23], Fmoc-Cl [18, 19, 23, 24], OPA [18, 25], Marfey's reagent [18], diethyl ethoxymethylenemalonate [23], and butanol [19]. Studies with on phenol applications focused on Dansyl-Cl [26], Dabsyl-Cl [21], and azo coupling reactions [27]. Due to their applications for both amines and aromatics, Dabsyl-Cl and Dansyl-Cl were selected. Fmoc-Cl was also selected as means of having an increased focus on the amine functionality of the neurotransmitters. A few analytical methods were considered based on previous literature: liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [18, 28]. Multidimensional high-performance liquid chromatography mass spectrometry (2D-HPLC-MS) has little to

no research regarding its validity for neurotransmitter applications and was chosen to be evaluated.

Multidimensional High-Performance Liquid Chromatography

Multidimensional high-performance liquid chromatography is liquid chromatography that combines multiple pumps in various configurations. Simpler configurations may only use two or three pumps, but more complex setups can use as many as six [30, 31]. The configuration for the experiment makes use of a three-pump design as seen in Figure 2. Two quaternary pumps, also called quaternary solvent managers (QSMs), act as the loader and diluter. A single binary pump, or binary solvent manager (BSM), is used to elute. Each dimension is designated as Dimension 1 (D1) and Dimension 2 (D2). The sample is trapped in the first dimension and eluted and separated in the second dimension with the aid of the eluter pump.

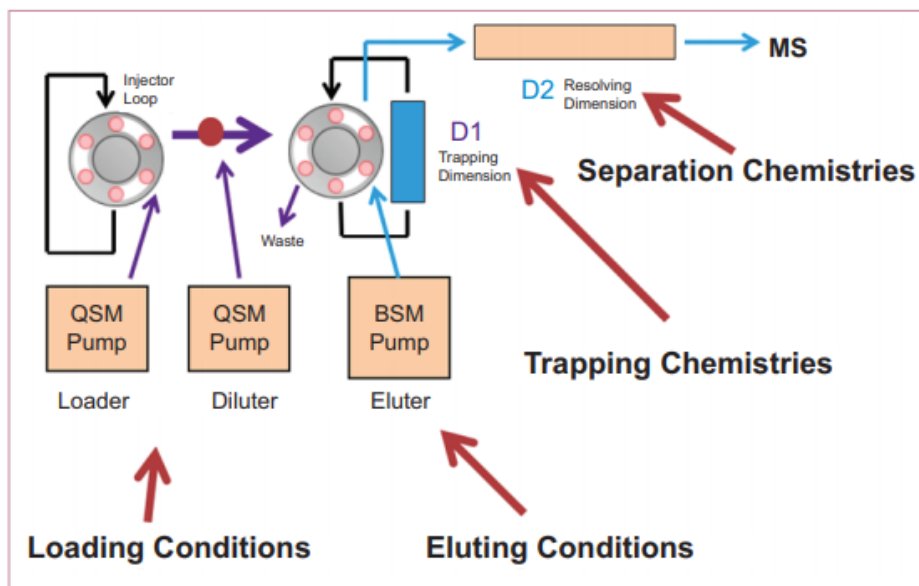


Figure 2. 2D-HPLC pump configuration with three pumps [30].

Multidimensional high-performance liquid chromatography is ideal for analysis due to its ability to be flexible in its configurations and the inclusion of an option to inject a large volume without distorting the peak shape due to backflush elution. [32, 33]. The increased number of pumps work in conjunction with using two dimensions to achieve chromatographic separation. The trapping dimension contains a large particle size which contains the target analyte within a narrow band. A second dimension contains smaller particles and utilizes back flush elution to move the analyte through the system [33]. The exact particle sizes will vary depending on the compounds being analyzed. By combining the two, the effect of breakthrough seen in large volume injection and the injection of high organic content is effectively eliminated [35]. The process decreases the need for long sample preparation by eliminating the need for an evaporation step in the extraction method [19, 35]. Separation, resolution, and limit of detection can be improved by altering the loading, trapping and eluting conditions to include different solvents at various pH values. Additional alterations are available such as changing the column chemistry can have a significant effects based on the sample's affinity to the column's specific functional group. The following experiment utilizes acetonitrile and methanol at pH 3 and 10 as well as aqueous phase at pH 3, 7, and 10 as the solvents. Several column chemistries included in the experiment are C8 and HLB for trapping and C18, BEH Phenyl, and HSS T3 for elution. The parameters listed were chosen for their frequency of use in laboratory settings as well as overall effectiveness for a variety of samples. With optimization of the 2D method, high precision and accuracy can be achieved as well as increased limit of detection and specificity [20, 21]. However, 2D-LC can be more expensive than traditional LC

methods and is seen as too complex for first-time users [30]. With adequate training the method is more accessible for those with little to no experience. The ability to have shorter run times increases the throughput and productivity of the instrument which can offset initial costs [30]. The benefits of multidimensional liquid chromatography make it ideal for the analysis of neurotransmitters and allows for the compounds to have a higher likelihood of being detected. The experiments described below seek to determine if chromatographic separation improves by combining the effects of derivatization and multidimensional liquid chromatography. If chromatography does improve, then pairing the two methods together could be a viable way of improving detection of neurotransmitters.

MATERIALS AND METHODS

Chemicals

Standards

Standards of dopamine, L-DOPA, serotonin, noradrenaline, tyrosine, tryptophan, and tyramine were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). A stock solution of each standard compound was made at 1.0 mg/mL in 80:20 acetonitrile (ACN) to water by measuring 10-20 mg of solid standard in a 20 mL glass vial and adding the corresponding amounts of ACN and water. If the solution was not completely dissolved, 100 μ L portions of concentrated HCl were added until completely dissolved and aided by sonication if necessary. Additional dilutions were completed in water, ACN, and MeOH separately to have standards at 1.0 μ g/mL (1 ppm) and 1.0 ng/mL (1 ppb).

Mobile Phase

HPLC-grade ACN and MeOH were purchase from Fisher Scientific (Pittsburg, Pennsylvania, USA). Organic and aqueous elution mobile phases were prepared by the addition of 0.5% FA in 2.0 L for low pH (pH 3) solutions and 0.5% NH_2OH in 2.0 L for high pH (pH 10) solutions. Loading mobile phase was prepared as aqueous solutions at pH 3 (2% FA), pH 7 (no buffer), and pH 10 (2% NH_2OH) in 5.0 L. Additional organic (MeOH) and aqueous mobile phase was made with 0.5% NH_4F in 2.0 L and 0.5% ammonium bicarbonate separately.

Derivatization Standards

Standards of Dansyl-Cl, Dabsyl-Cl, and FMOC-Cl were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and diluted to 1.0 mg/mL in ACN. Glycine was also obtained from Sigma and diluted to 0.1 mg/mL in deionized water. Buffers for the derivatization process were mixed in ACN (boric acid – pH 8.5, sodium borate – pH 9.5, sodium bicarbonate – pH 10.5, NaOH – pH 14). HPLC grade acetone and ethyl acetate (EA) were purchased from Fisher Scientific (Pittsburg, Pennsylvania, USA).

Instrumentation

LC Setup

LC analysis was completed on a Waters Acquity UPLC Class system (Milford, Massachusetts, USA) in a two-dimensional configuration with two quaternary solvent managers (QSMs) used for loading and dilution, one binary solvent manager (BSM) used for elution, autosampler, and column manager. Two columns were examined for trapping: Waters Acquity UPLC C8 (2.1mm x 30mm, 10 μ m bead size) and Waters Acquity HLB (2.1mm x 30mm, 20 μ m bead size). Three columns were examined for elution: Waters Acquity HSS T3 (2.1mm x 100mm, 1.7 μ m bead size), Waters Acquity UPLC BEH C18 (2.1mm x 100mm, 1.7 μ m bead size), and Waters Acquity BEH Phenyl (2.1mm x 100mm, 1.7 μ m bead size). Injection volume was 20 μ L. Dilution flow rate was set at 2.0 ml/min and loading flow rate at 0.1 mL/min. Elution occurred at 0.5 ml/min with the organic gradient as follows: 0-3 min (5% B), 3-8 min (5% to 95% B), 8-9 min (95% B), 9-9.5 min (95% to 5% B), 9.5-10 min (5% B).

MS Setup

MS analysis was completed on a Waters Acquity UPLC Class system connected to a Waters Xevo TQ-S with Intellistart (Milford, Massachusetts, USA) set in positive ESI mode. Capillary voltage was set at 3.0 kV, cone voltage at 30 V, and source offset at 60V determined offline. Desolvation temperature was set at 450°C and desolvation flow at 1100 L/hr. Cone flow was set at 150 L/hr.

Derivatization Process

Before starting the process, a mixture of the neurotransmitters was made and was diluted in acetonitrile to a final concentration of 1.0 µg/mL. Derivatization with each compound (Dabsyl-Cl, Dansyl-Cl, FMOC-Cl) was completed as follows. In a glass tube 800 µL of deionized water was added followed by 580 µL of one organic solvent (ACN, acetone, or ethyl acetate). Then 200 µL of buffer (boric acid – pH 8.5, sodium borate – pH 9.5, sodium bicarbonate – pH 10.5, NaOH – pH 14) was introduced to maintain the pH of the reaction. 200 µL of the neurotransmitter mix (1.0 µg/mL) was added. Finally, 20 µL of the derivatization compound (1.0 mg/mL) was added. Each vial was topped with aluminum foil and vortexed for three to five seconds then heated at 60° C for thirty minutes. Once removed the heat, the aluminum foil was removed and 200 µL of glycine at 0.1 mg/mL. For this project samples were made containing each combination of derivatization compound, buffer pH, and organic solvent.

RESULTS AND DISCUSSION

Underivatized Compounds

The analysis of neurotransmitters was first completed by examining the data before derivatization. It would determine the overall baseline. Once the data for spectrometry and chromatography was collected, a decision needed to be reached as to whether or not the derivatization process would be required.

Spectrometry Data

Mass spectrometry data was collected to selected and optimize two multiple reaction monitoring (MRM) transitions for quantification and confirmation using a process adapted from several studies utilizing 2D technology [15, 32, 33, 35, 36]. Due to the use of ESI+ ionization mode, each neurotransmitter was protonated, and the resulting ions are in their $[M+H]^+$ state. Precursor ions were determined by infusion using MS scan mode at pH 3, 7, and 10. Each scan was captured to confirm the presence of the specific neurotransmitter and later focused on the apex of the peak to determine the decimal value. In the resulting scan for dopamine (Figure 3), the protonated mass of 154 was seen at pH 3 and 7 but is not shown at pH 10. Scans for L-DOPA in Figure 4 show similar difficulties with the ion of m/z 197 or 198 only appearing at pH 3 and 7. Both sets of scans for serotonin (Figure 5) and tyrosine (Figure 6) show peaks for their respective ions, m/z 177 and 182, at all three pH values.

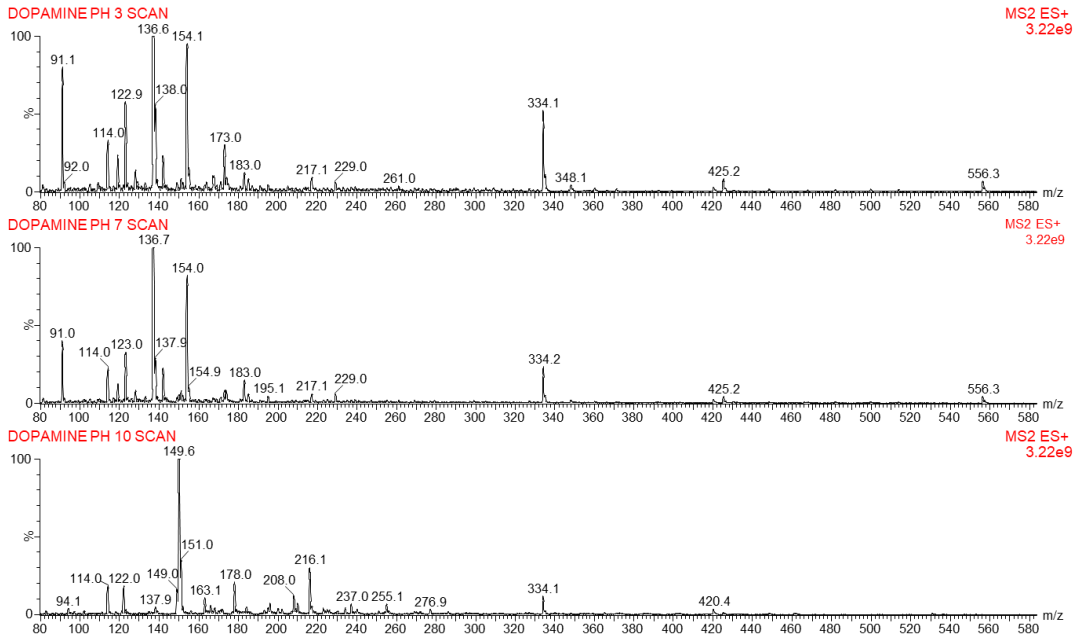


Figure 3. MS Scan of underivatized dopamine at pH 3, 7, and 10.

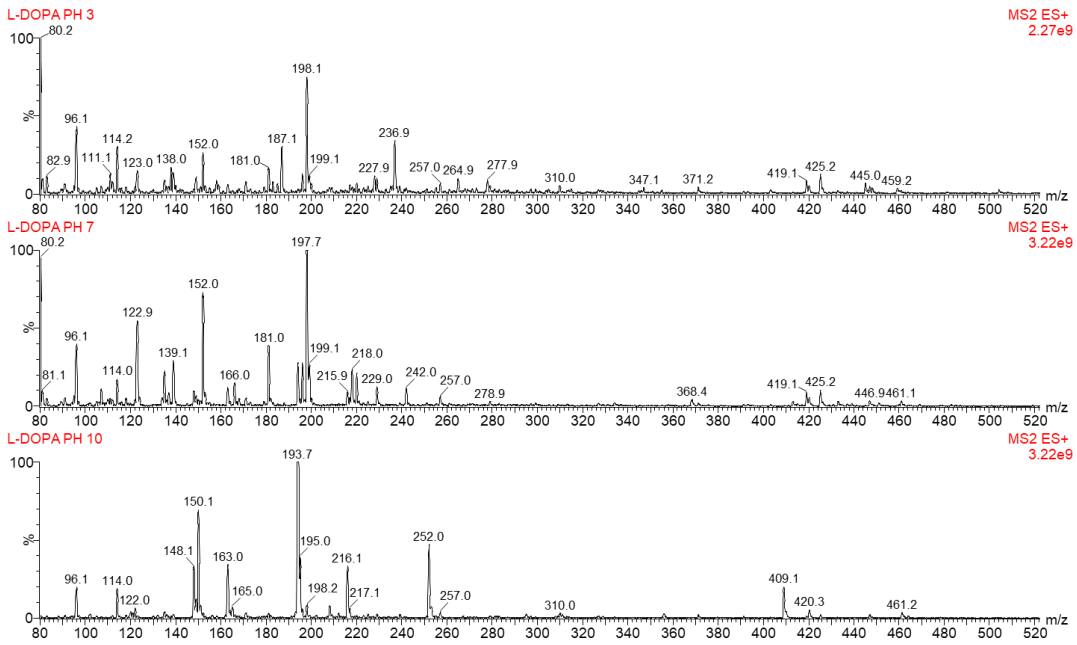


Figure 4. MS Scan of underivatized L-DOPA at pH 3, 7, And 10.

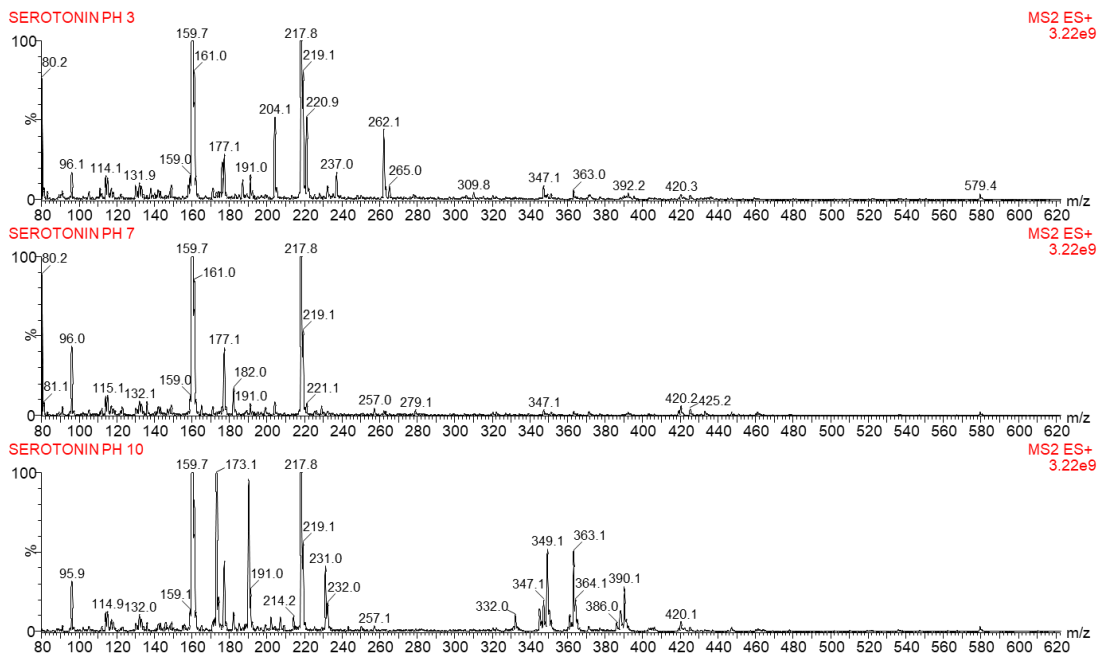


Figure 5. MS Scan of underivatized serotonin at pH 3, 7, and 10.

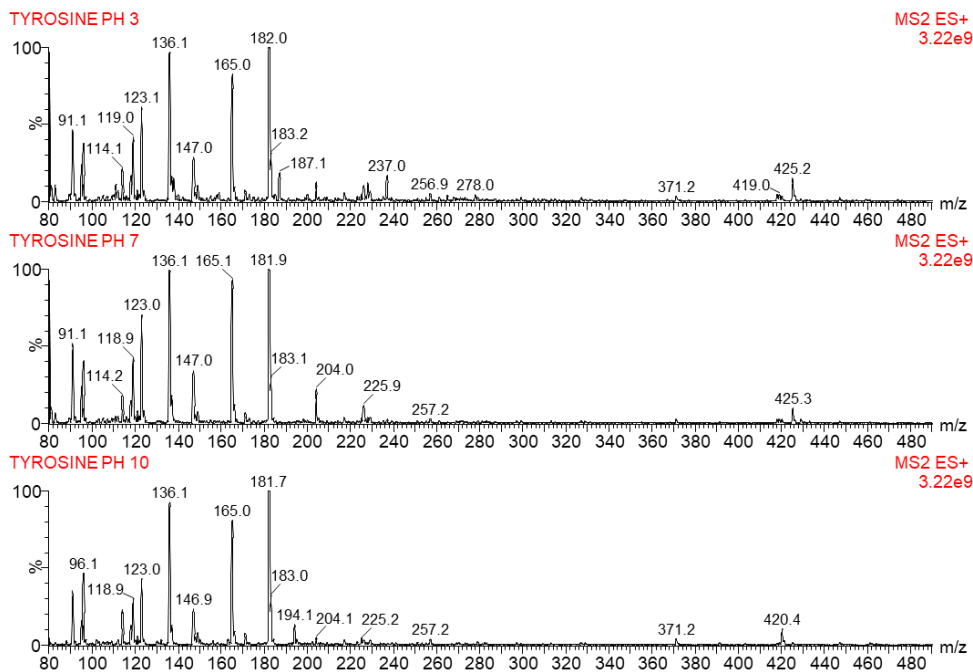


Figure 6. MS Scan of underivatized tyrosine at pH 3, 7, and 10.

Scans of tryptophan (Figure 7) show intense peaks at the desired ion value of m/z 205, but also shows significant peaks at 188 m/z $[M-H_2O]$ for all three pH values and at m/z 226 $[M+Na]$ at pH 10. Each of the other neurotransmitters analyzed in the experiment do not correlate to the foreign ion values and can be eliminated as the reason for the peaks. Noradrenaline was the most difficult neurotransmitter for the MS scan to detect. Figure 8 shows the desired ion of m/z 171 at pH 3 and 7, but the peaks are weak compared to peaks around m/z 420 and 425 which could be carryover from a previous sample.

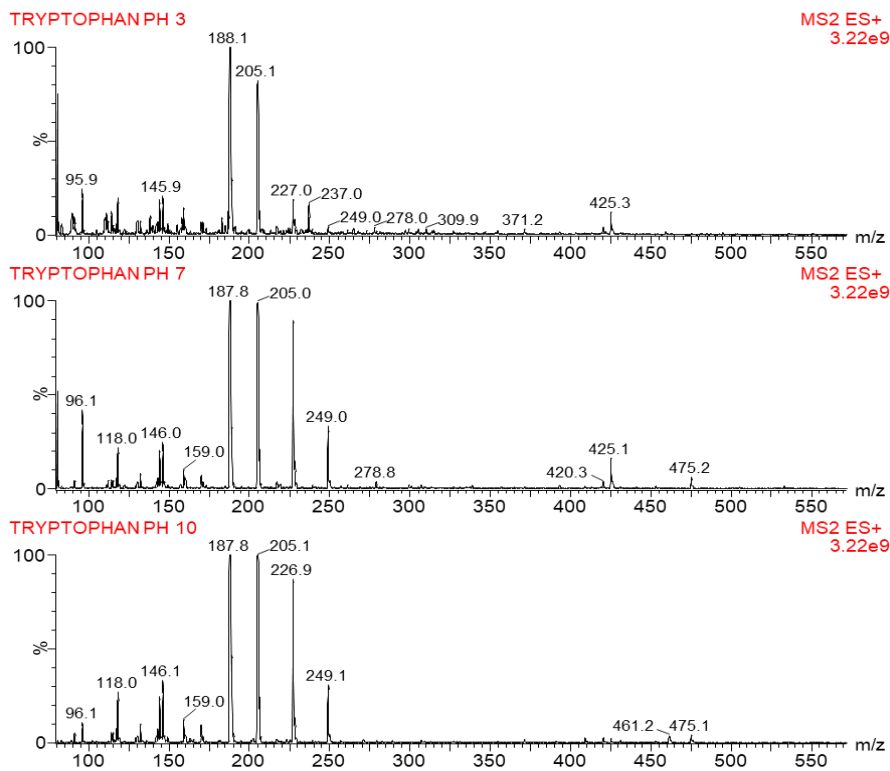


Figure 7. MS Scan of underivatized tryptophan at pH 3, 7, and 10.

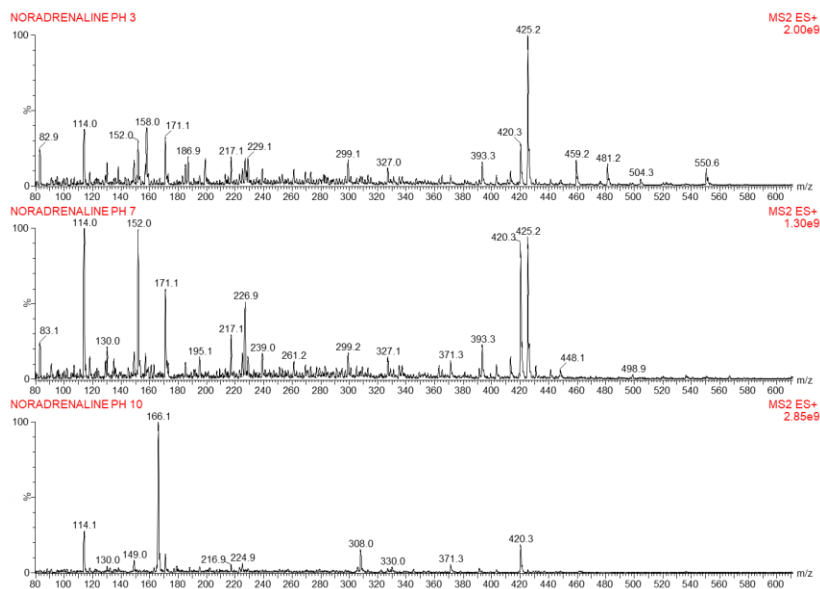


Figure 8. MS Scan of underivatized noradrenaline at pH 3, 7, and 10.

Once the precursor ions were determined, the next step was to determine the product ion values and their respective collision induced dissociation (CID) energy values. Production of product ions was completed using infusion under MS/MS scan mode with acquisitions at each CID increasing the value by five each time. CID was raised until the precursor ion peak disappeared. The highest CID level for this portion was 25 or 30 depending on the neurotransmitter. Spectra only show values of CID 5 to CID 25. Once each spectra was acquired, the two most prominent peaks were selected as product ions and their corresponding CID value was determined when the ions were at the highest intensity. Analysis of the apex of individual peaks at the selected CID value determined the decimal value. Resulting values of MS and MS/MS scans can be seen in Table 1. Selected CID values were 25 or lower which shows that underivatized neurotransmitters do not need a significant amount of energy to fragment.

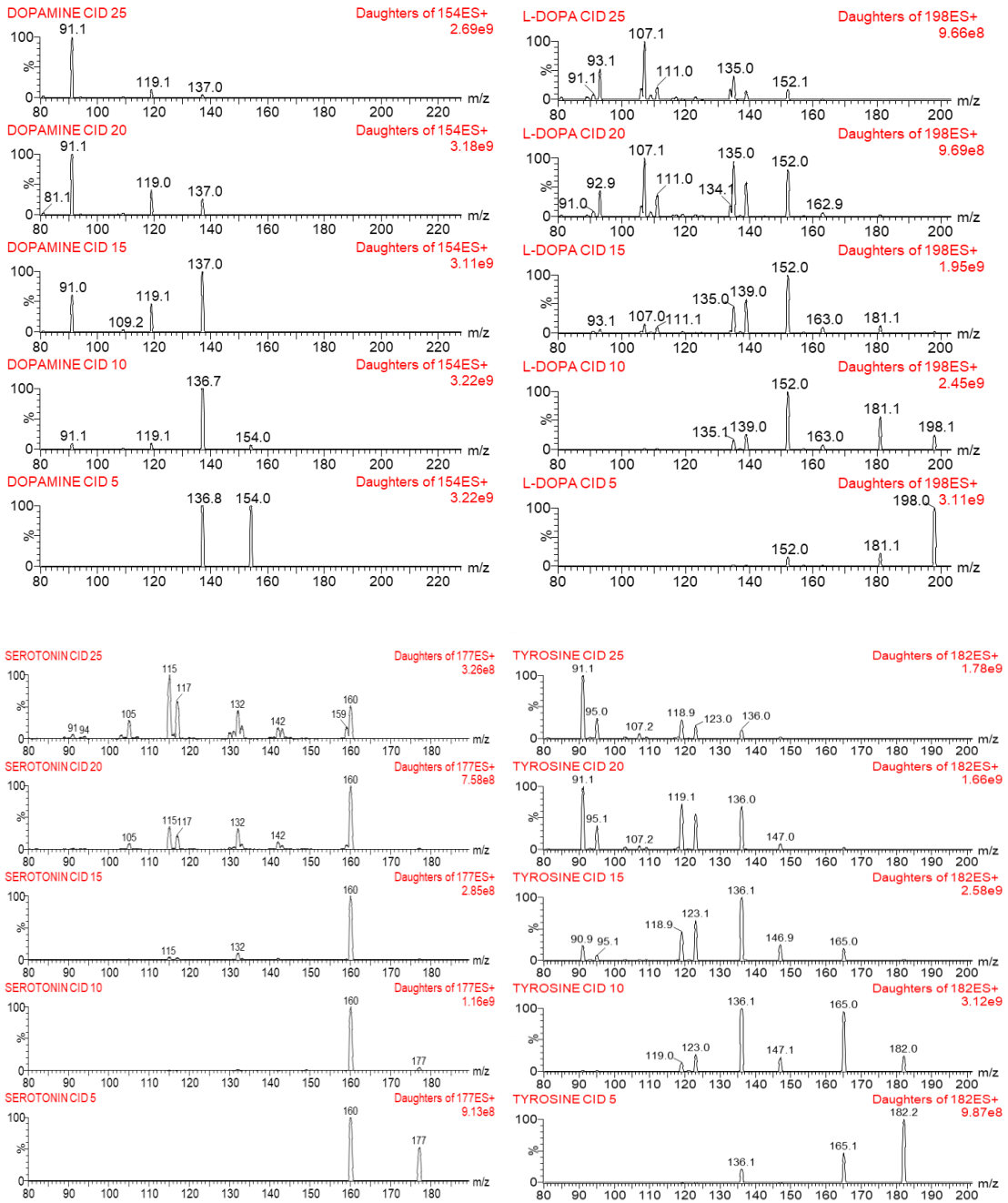


Figure 9. MS/MS scans of dopamine, L-DOPA, serotonin, and tyrosine from CID 5 to CID

25.

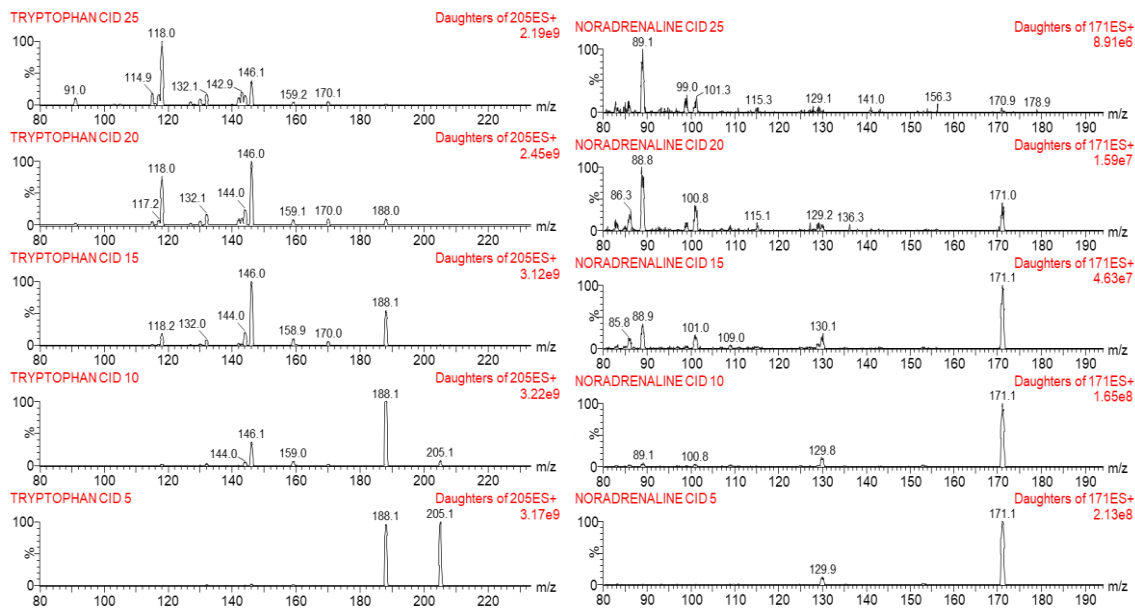


Figure 10. MS/MS scans of tryptophan and noradrenaline from CID 5 to CID 25.

Table 1. MRMs - Precursor and product ion values and corresponding CID energy values.

Neurotransmitter	Precursor Ion	Product Ion 1	CID 1	Product Ion 2	CID 2
Dopamine	154.5	137.1	10	91.1	20
L-DOPA	198.2	152.1	10	107.1	20
Serotonin	177.2	160.1	20	142.2	20
Tyrosine	182.1	136.2	10	91.1	25
Tryptophan	205.2	146.1	15	118.1	25
Noradrenaline	171.4	101.1	15	89.1	15

Chromatography Data

Once the MRMs were developed, the next step was to determine the quality of chromatography for each neurotransmitter. The goal was to optimize different parameters on both dimensions. The first dimension has a focus on the retention strength of the trap column as well as the loading pH to effectively retain the sample in a narrow band. For the second dimension the priority is elution which relies on the pH and polarity of the elution solvent. There are a wide variety of potential options for each parameter, but the number of combinations would be too large for the scope of the project. To keep the process streamlined, the first dimension was limited to the three most common loading pHs and trap column chemistries. The second dimension focused on two elution pHs and two elution solvents. Each combination is displayed in a 6x6 grid format shown in Figure 11 and the corresponding method number. Initial tests of the C18 trap column gave unreadable signal and was further omitted from the study. Analysis was completed using 20 μ L injections of a 1.0 μ g/mL mix of all of the neurotransmitters.

Loading pH			
pH 10	1	2	3
pH 7	4	5	6
pH 3	7	8	9
	C8	C18	HLB
	Trap Column Chemistry		

Loading pH			
pH 10	10	11	12
pH 7	13	14	15
pH 3	16	17	18
	C8	C18	HLB
	Trap Column Chemistry		

Loading pH			
pH 10	19	20	21
pH 7	22	23	24
pH 3	25	26	27
	C8	C18	HLB
	Trap Column Chemistry		

Loading pH			
pH 10	28	29	30
pH 7	31	32	33
pH 3	34	35	36
	C8	C18	HLB
	Trap Column Chemistry		

Figure 11. 6x6 grid with 36 methods for optimization.

Results of the 6x6 grid methods for each elution column are shown in Figures 13-17. Each neurotransmitter was prepared in water, ACN, or MeOH in preparation for potential use with solid phase extraction (SPE). The three colors represent the quality of chromatography. Green indicates that the resulting peak was Gaussian in shape and notes the corresponding intensity of the peak as well as the presence of multiple peaks (MP). Yellow implies peak distortion and notes the appearance of the distortion such as broad, tailing, fronting, or split peaks. Red denotes that the peak is indistinguishable either from noise or the overall chromatogram is too distorted. Examples of each color can be seen in Figure 12. Within the grid results, it can be seen that the majority of resulting chromatograms fell into the red or yellow category for all of the elution columns. The BEH C18 column with a pH 10 elution in ACN showed the largest number of green boxes overall, but across the board noradrenaline and serotonin could not be distinguished using this method.

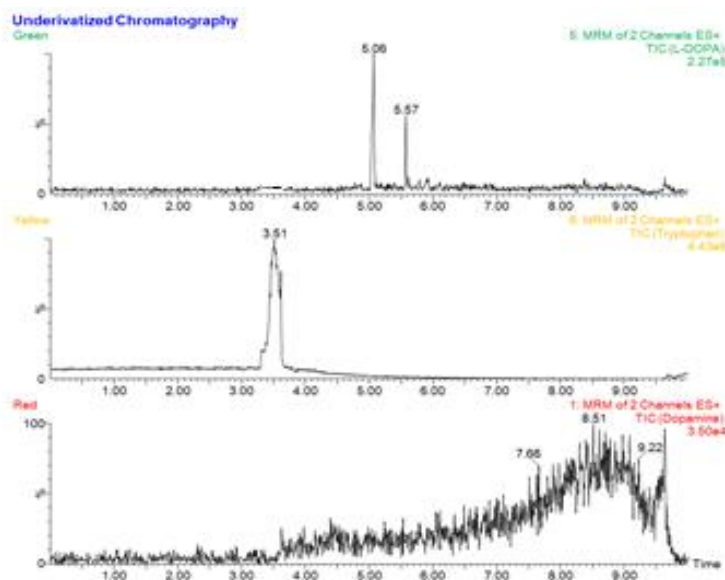


Figure 12. Chromatography Color Examples. Green on top, Yellow in middle, Red on bottom.

HSS T3 ACN pH 3 elution									
	1	2	3	4	5	6	7	8	9
Dopamine (Water)									
Dopamine (ACN)									
Dopamine (MeOH)									
Noradrenaline (Water)									
Noradrenaline (ACN)									
Noradrenaline (MeOH)									
Serotonin (Water)									
Serotonin (ACN)									
Serotonin (MeOH)									
Tyrosine (Water)							e5 MP		e5 MP
Tyrosine (ACN)							e5 MP		e5 MP
Tyrosine (MeOH)							e5 MP		e5 MP
L-DOPA (Water)	e5		e5						
L-DOPA (ACN)	e5		e5						
L-DOPA (MeOH)	e6		e6						
Tryptophan (Water)	broad		broad			broad			
Tryptophan (ACN)	broad		broad			broad			
Tryptophan (MeOH)	broad		broad			broad			

HSS T3 MeOH pH 3 elution									
	19	20	21	22	23	23	25	26	27
Dopamine (Water)									
Dopamine (ACN)									
Dopamine (MeOH)									
Noradrenaline (Water)									
Noradrenaline (ACN)									
Noradrenaline (MeOH)									
Serotonin (Water)									
Serotonin (ACN)									
Serotonin (MeOH)									
Tyrosine (Water)									
Tyrosine (ACN)									
Tyrosine (MeOH)									
L-DOPA (Water)									
L-DOPA (ACN)									
L-DOPA (MeOH)									
Tryptophan (Water)									
Tryptophan (ACN)									
Tryptophan (MeOH)									

Figure 13. 6x6 grid results HSS T3 elution. High pH elution for ACN (method 10-18) and MeOH (method 28-36) were not processed due to time constraints.

BEH C18 ACN pH 3 elution									
	1	2	3	4	5	6	7	8	9
Dopamine (Water)	Red	Red	Red	Red	Red	Red	e5	Green	broad
Dopamine (ACN)	Red	Red	Red	Red	Red	Red	e5	Green	broad
Dopamine (MeOH)	Red	Red	Red	Red	Red	Red	e5	Green	broad
Noradrenaline (Water)	Red	Red	Red	Red	Red	Red	Red	Red	Red
Noradrenaline (ACN)	Red	Red	Red	Red	Red	Red	Red	Red	Red
Noradrenaline (MeOH)	Red	Red	Red	Red	Red	Red	Red	Red	Red
Serotonin (Water)	Red	Red	Red	Red	Red	Red	broad	Yellow	broad
Serotonin (ACN)	Red	Red	Red	Red	Red	Red	broad	Yellow	broad
Serotonin (MeOH)	Red	Red	Red	Red	Red	Red	broad	Yellow	broad
Tyrosine (Water)	Red	Red	Red	Red	Red	broad	Red	Red	Red
Tyrosine (ACN)	Red	Red	Red	Red	Red	broad	Red	Red	Red
Tyrosine (MeOH)	Red	Red	Red	Red	Red	broad	Red	Red	Red
L-DOPA (Water)	Red	Red	Red	e5	Green	e5	Green	Green	Red
L-DOPA (ACN)	Red	Red	Red	e5	Green	e5	Green	Green	Red
L-DOPA (MeOH)	Red	Red	Red	e5	Green	e5	Green	Green	Red
Tryptophan (Water)	Red	Red	Red	Red	Red	broad	Red	Red	broad
Tryptophan (ACN)	Red	Red	Red	Red	Red	broad	Red	Red	broad
Tryptophan (MeOH)	Red	Red	Red	Red	Red	broad	Red	Red	broad

BEH C18 ACN pH 10 elution									
	10	11	12	13	14	15	16	17	18
Dopamine (Water)	Red	Red	Red	e5	Green	e7	Green	e6	Green
Dopamine (ACN)	Red	Red	Red	e5	Green	e6	Green	e6	Green
Dopamine (MeOH)	Red	Red	Red	e5	Green	e6	Green	e6	Green
Noradrenaline (Water)	Red	Red	Red	Red	Red	Red	Red	Red	Red
Noradrenaline (ACN)	Red	Red	Red	Red	Red	Red	Red	Red	Red
Noradrenaline (MeOH)	Red	Red	Red	Red	Red	Red	Red	Red	Red
Serotonin (Water)	Red	Red	Red	Red	Red	Red	Red	Red	broad
Serotonin (ACN)	Red	Red	Red	Red	Red	Red	Red	Red	broad
Serotonin (MeOH)	Red	Red	Red	Red	Red	Red	Red	Red	broad
Tyrosine (Water)	e6	Green	e6	Green	e7	Green	e6	Green	Red
Tyrosine (ACN)	e6	Green	e6	Green	e7	Green	e6	Green	Red
Tyrosine (MeOH)	e6	Green	e6	Green	e7	Green	e6	Green	Red
L-DOPA (Water)	e5	Green	e5	Green	e5	Green	e5	Green	Red
L-DOPA (ACN)	e5	Green	e5	Green	e5	Green	e5	Green	Red
L-DOPA (MeOH)	e5	Green	e5	Green	e5	Green	e5	Green	Red
Tryptophan (Water)	e7	Green	e8	Green	e7	Green	e8	Green	e6
Tryptophan (ACN)	e7	Green	e8	Green	e7	Green	e8	Green	e6
Tryptophan (MeOH)	e7	Green	e8	Green	e7	Green	e8	Green	e6

Figure 14. 6x6 grid results BEH C18 ACN elution.

BEH C18 MeOH pH 3 elution										
	19	20	21	22	23	24	25	26	27	
Dopamine (Water)	e6		broad				e6		broad	
Dopamine (ACN)	e6		broad				e6		broad	
Dopamine (MeOH)	e6		broad				e6		broad	
Noradrenaline (Water)										
Noradrenaline (ACN)										
Noradrenaline (MeOH)										
Serotonin (Water)						broad			broad	
Serotonin (ACN)						broad			broad	
Serotonin (MeOH)						broad			broad	
Tyrosine (Water)			broad							
Tyrosine (ACN)			broad							
Tyrosine (MeOH)			broad							
L-DOPA (Water)			broad							
L-DOPA (ACN)			broad							
L-DOPA (MeOH)			broad							
Tryptophan (Water)	broad		broad			broad				
Tryptophan (ACN)	broad		broad			broad				
Tryptophan (MeOH)	broad		broad			broad				

BEH C18 MeOH pH 10 elution										
	28	29	20	31	32	33	34	35	36	
Dopamine (Water)										
Dopamine (ACN)										
Dopamine (MeOH)										
Noradrenaline (Water)										
Noradrenaline (ACN)										
Noradrenaline (MeOH)										
Serotonin (Water)						broad	e6		broad	
Serotonin (ACN)						broad	e6		broad	
Serotonin (MeOH)						broad	e6		broad	
Tyrosine (Water)										
Tyrosine (ACN)										
Tyrosine (MeOH)										
L-DOPA (Water)			e5							
L-DOPA (ACN)			e5							
L-DOPA (MeOH)			e5							
Tryptophan (Water)	e6		broad	e5		broad	e5		broad	
Tryptophan (ACN)	e6		broad	e5		broad	e5		broad	
Tryptophan (MeOH)	e6		broad	e5		broad	e5		broad	

Figure 15. 6x6 grid results BEH C18 MeOH elution pH 3 and 10.

BEH C18 MeOH NH4F elution									
	19	20	21	22	23	24	25	26	27
Dopamine (Water)									
Dopamine (ACN)									
Dopamine (MeOH)									
Noradrenaline (Water)									
Noradrenaline (ACN)									
Noradrenaline (MeOH)									
Serotonin (Water)									
Serotonin (ACN)									
Serotonin (MeOH)									
Tyrosine (Water)							e5		e5
Tyrosine (ACN)							e5		e5
Tyrosine (MeOH)							e5		e5
L-DOPA (Water)									
L-DOPA (ACN)									
L-DOPA (MeOH)									
Tryptophan (Water)									
Tryptophan (ACN)									
Tryptophan (MeOH)									

BEH C18 MeOH NH4Bicarbonate elution									
	28	29	20	31	32	33	34	35	36
Dopamine (Water)									
Dopamine (ACN)									
Dopamine (MeOH)									
Noradrenaline (Water)									
Noradrenaline (ACN)									
Noradrenaline (MeOH)									
Serotonin (Water)									
Serotonin (ACN)									
Serotonin (MeOH)									
Tyrosine (Water)									
Tyrosine (ACN)									
Tyrosine (MeOH)									
L-DOPA (Water)	e5		e5						
L-DOPA (ACN)	e5		e5						
L-DOPA (MeOH)	e5		e5						
Tryptophan (Water)									
Tryptophan (ACN)									
Tryptophan (MeOH)									

Figure 16. 6x6 grid results BEH C18 MeOH elution NH₄F and Ammonium bicarbonate.

BEH Phenyl MeOH NH4F elution									
	19	20	21	22	23	24	25	26	27
Dopamine (Water)									
Dopamine (ACN)									
Dopamine (MeOH)									
Noradrenaline (Water)									
Noradrenaline (ACN)									
Noradrenaline (MeOH)									
Serotonin (Water)									
Serotonin (ACN)									
Serotonin (MeOH)									
Tyrosine (Water)							e5	e5	
Tyrosine (ACN)							e5	e5	
Tyrosine (MeOH)							e5	e5	
L-DOPA (Water)									
L-DOPA (ACN)									
L-DOPA (MeOH)									
Tryptophan (Water)									
Tryptophan (ACN)									
Tryptophan (MeOH)									

BEH Phenyl MeOH NH4Bicarbonate elution									
	28	29	30	31	32	33	34	35	36
Dopamine (Water)									
Dopamine (ACN)									
Dopamine (MeOH)									
Noradrenaline (Water)									
Noradrenaline (ACN)									
Noradrenaline (MeOH)									
Serotonin (Water)									
Serotonin (ACN)									
Serotonin (MeOH)									
Tyrosine (Water)									
Tyrosine (ACN)									
Tyrosine (MeOH)									
L-DOPA (Water)			e5						
L-DOPA (ACN)			e5						
L-DOPA (MeOH)			e5						
Tryptophan (Water)									
Tryptophan (ACN)									
Tryptophan (MeOH)									

Figure 17. 6x6 grid results BEH Phenyl MeOH elution NH₄F and Ammonium bicarbonate.

Derivatized Compounds

Due to poor chromatography of the underivatized samples and testing of each variable, the choice was made to explore the option of modifying the structure to add some hydrophobicity to the molecule. The modification would be done through the process of derivatization. For the derivatization, three agents were selected: Dabsyl-Cl, Dansyl-Cl, and FMOC-Cl. Initial testing of the FMOC-Cl solution showed no change in the overall quality of the chromatography and was dismissed as a potential derivative. Therefore, the focus was shifted onto the other two compounds.

Selection of Method

Once Dabsyl-Cl and Dansyl-Cl were selected, preparation of the standards were made at 1 mg/mL in ACN. Since the literature explained that Dabsyl-Cl and Dansyl-Cl could interact with phenols and amine functional groups, an initial examination was done to determine where on the molecule the derivatization agent would attach [18-23]. It was found that the agents would bind to primary and secondary amines through the loss of Cl on the agent and H⁺ from the amine. Structure of each agent and example binding locations are shown in Figure 18. The derivatization process was developed by adapting several previous studies that included the use of buffers at pH 8.5, 9.5, and 10.5 [18, 22, 26]. Since dopamine and other neurotransmitters are sensitive to high pH, another buffer of pH 14 was included. Tyramine, a compound structurally similar to dopamine, was included in the study as an internal standard for the neurotransmitter mix used for chromatography.

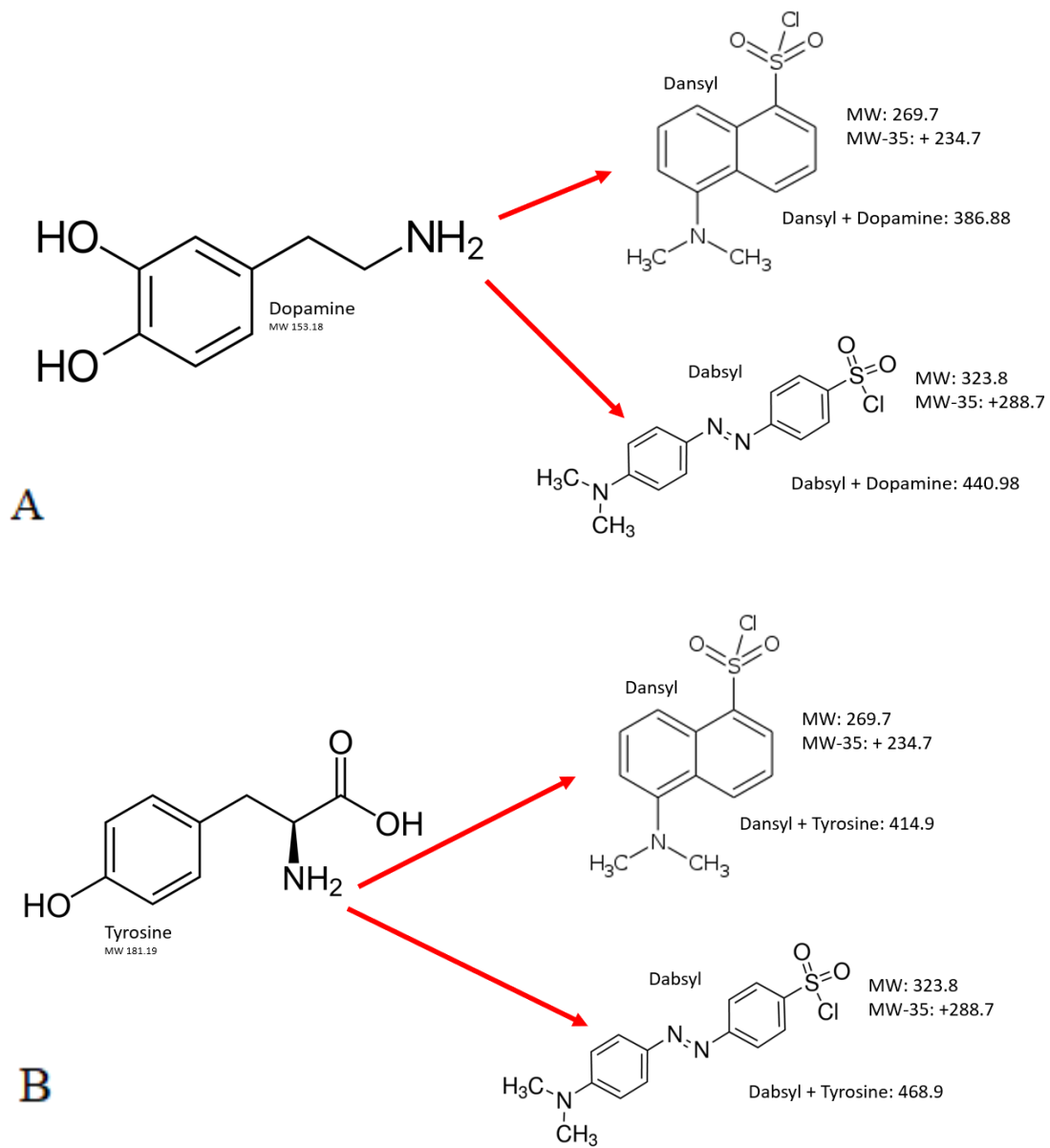


Figure 18. Dansyl and Dabsyl binding. A – primary amine, B – secondary amine.

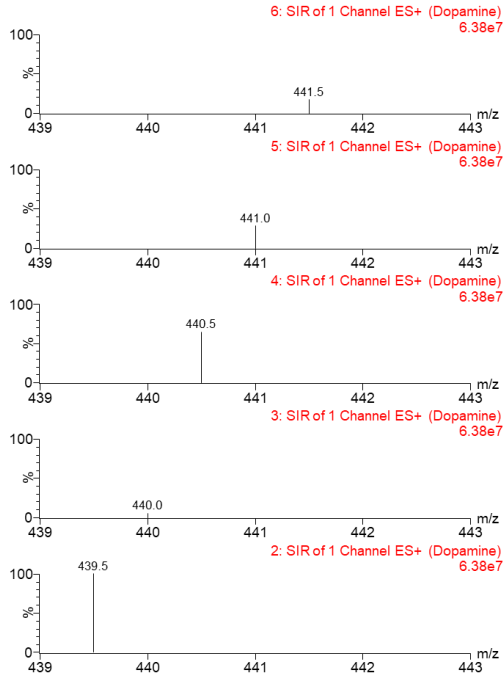
Spectrometry Data

Spectrometric data was collected in LC/MS mode instead of infusion as the concentration of the analytes were too low to be seen. Scans were collected to determine the precursor ion followed by Selected Ion Recording (SIR) scans to determine the decimal value. Initial precursor values for Dabsyl and Dansyl are shown in Table 2. SIR scans were taken between 2 amu above and below the calculated value in 0.5 amu increments. Product ions were determined by daughter scans taken at the SIR value with the highest intensity and one increment above. The final SIR value was determined by the one with the most intense product ions. SIR scans for Dabsyl are shown in Figures 19 and 20 and corresponding daughter scans are in Figure 21 and 22. Dansyl SIR scans are in Figure 23 and 24 and daughter scans are in Figure 25 and 26.

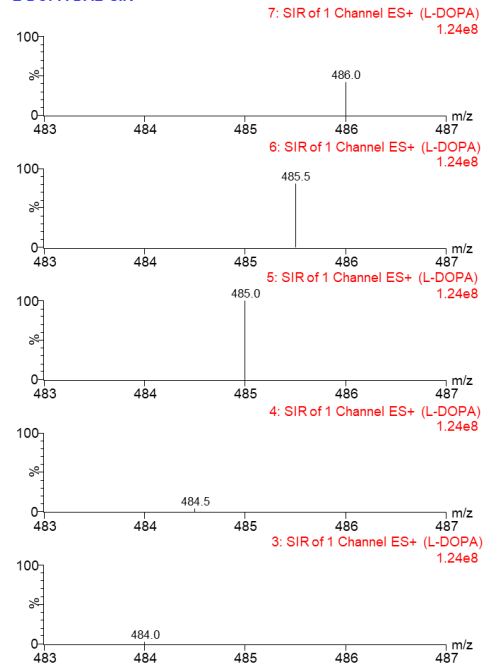
Table 2. Calculated molecular weight of neurotransmitters before and after derivatization.

Neurotransmitter	Molecular Weight (Underivatized)	MW of Derivatized Compound (Dabsyl)	MW of Derivatized Compound (Dansyl)
Dopamine	153.2	440.9	386.9
L-DOPA	197.2	484.9	430.9
Serotonin	176.2	463.9	409.9
Tyrosine	181.2	468.9	414.9
Tryptophan	204.2	491.9	437.9
Noradrenaline	169.2	456.9	402.9
Tyramine	137.2	424.9	370.9

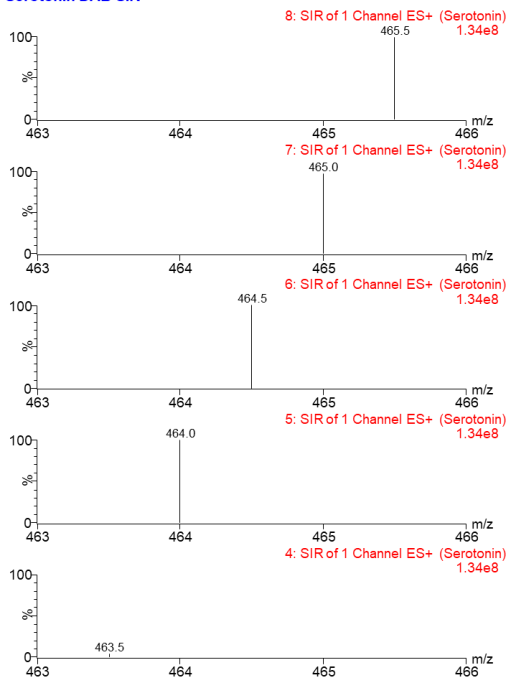
Dopamine DAB SIR



L-DOPA DAB SIR



Serotonin DAB SIR



Tyrosine DAB SIR

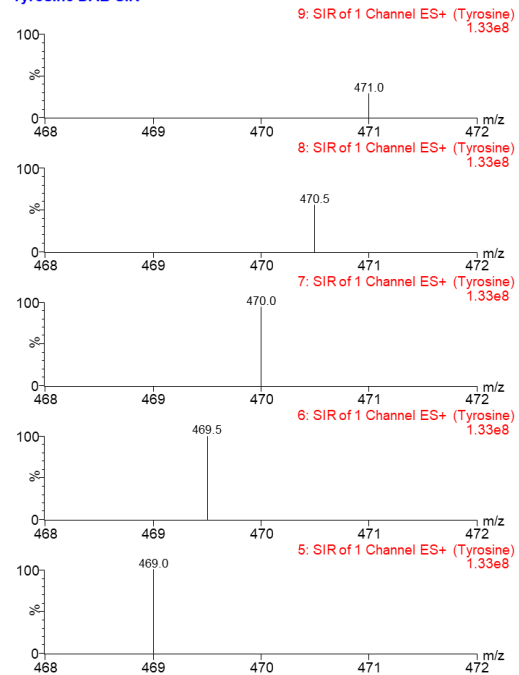
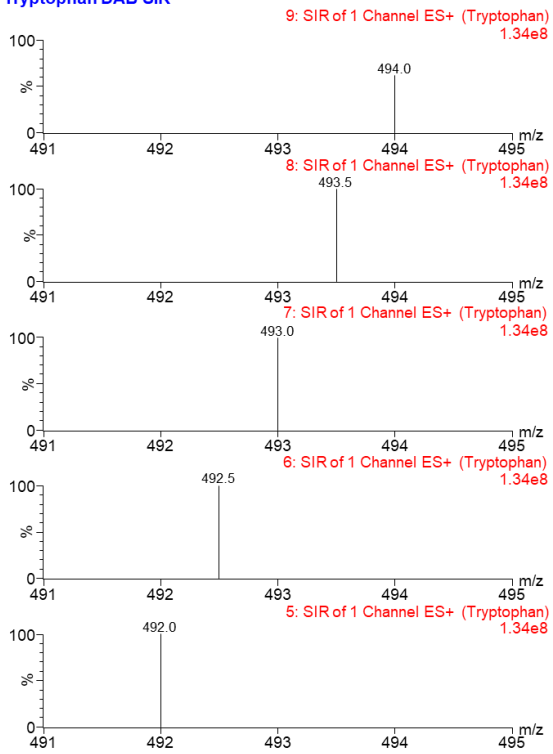
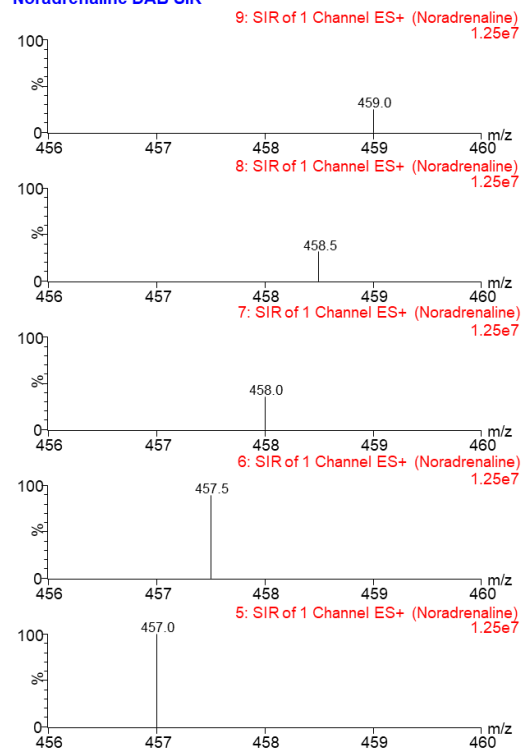


Figure 19. Dabsyl SIR scans for dopamine, L-DOPA, serotonin, and tyrosine.

Tryptophan DAB SIR



Noradrenaline DAB SIR



Tyramine DAB SIR

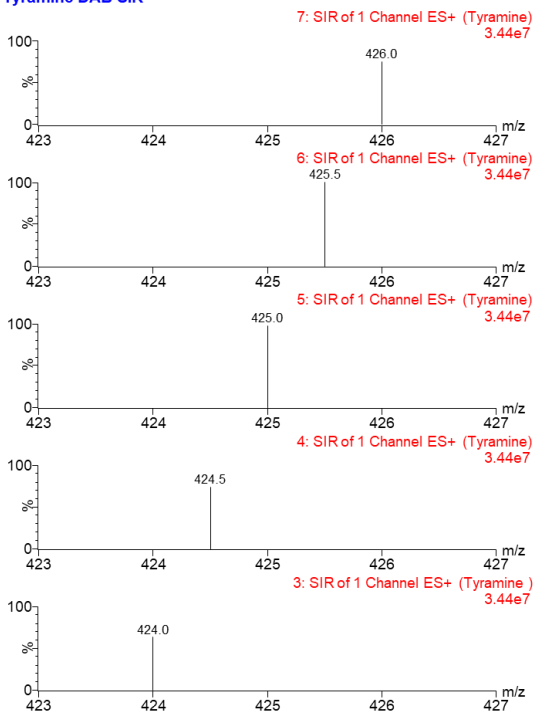
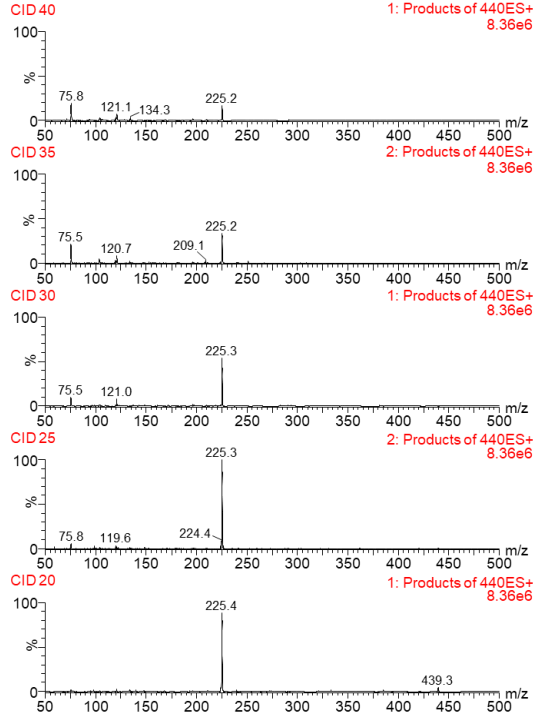
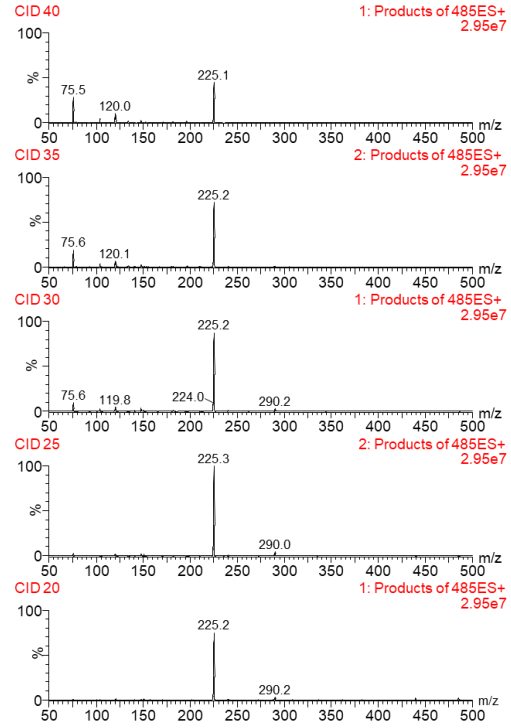


Figure 20. Dabsyl SIR scans for tryptophan, noradrenaline, and tyramine.

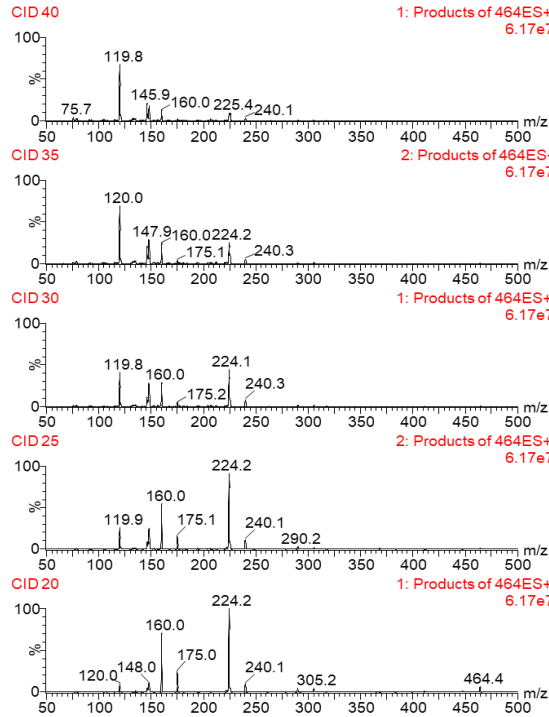
Dopamine DAB Product Ions



L-DOPA DAB Product Ions



Serotonin DAB Product Ions



Tyrosine DAB Product Ions

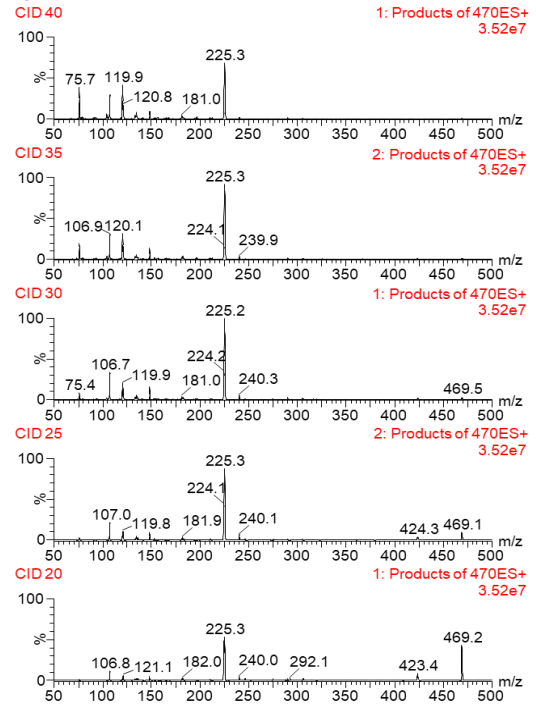
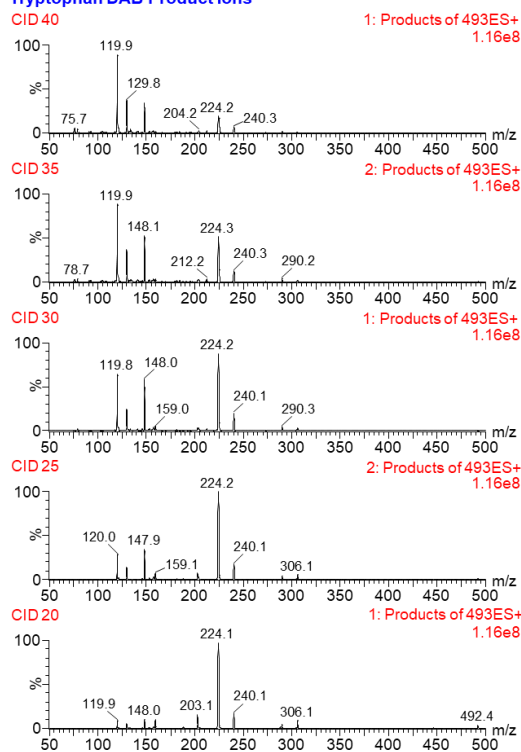
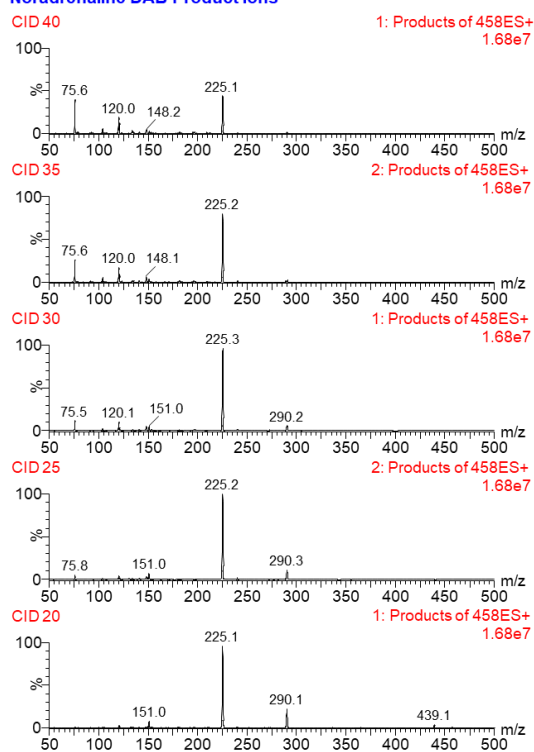


Figure 21. Dabsyl Product Ion scans for dopamine, L-DOPA, Serotonin, and tyrosine.

Tryptophan DAB Product Ions



Noradrenaline DAB Product Ions



Tyramine DAB Product Ions

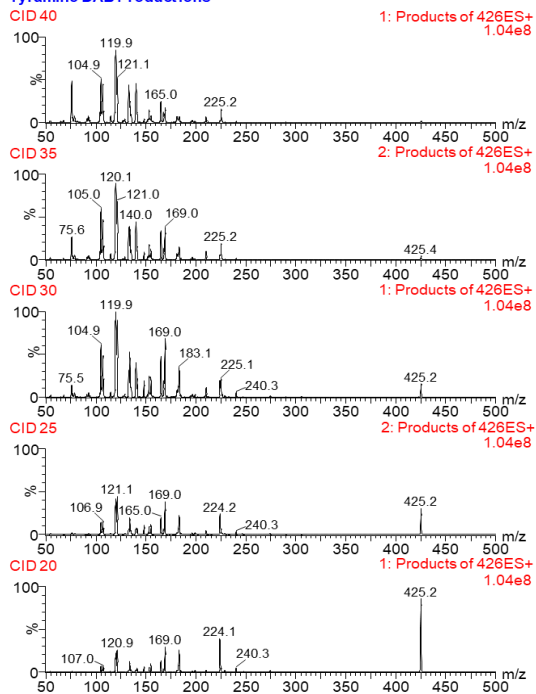


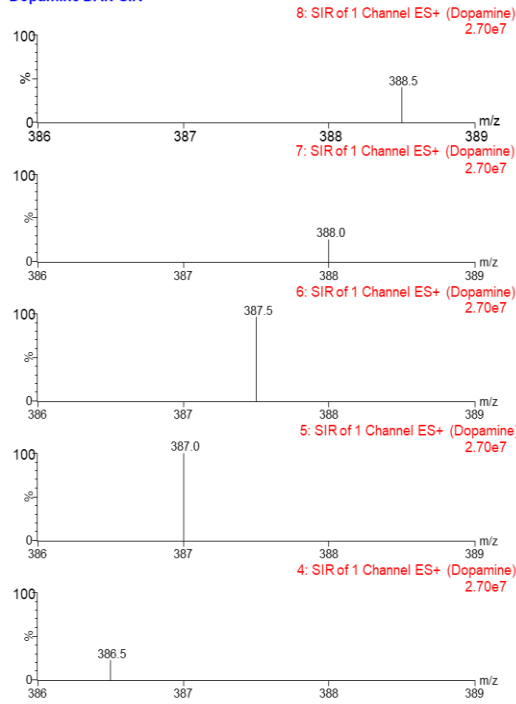
Figure 22. Dabsyl Product Ion scans for tryptophan, noradrenaline, and tyramine.

Once product ion scans were acquired, the ion values were used to create MRMs (Table 3 and 4). Each ion was analyzed individually to determine which would be the quantitative and qualitative transition. One major difference seen here in comparison to the underivatized compounds was that the ion values were almost identical for all compounds in both derivatization methods when run on the same day. Additionally, the average CID value was much higher with a minimum of CID 20. As the compounds were much larger with the addition of the derivatization agent, it would be logical to assume more energy is needed to cause fragmentation.

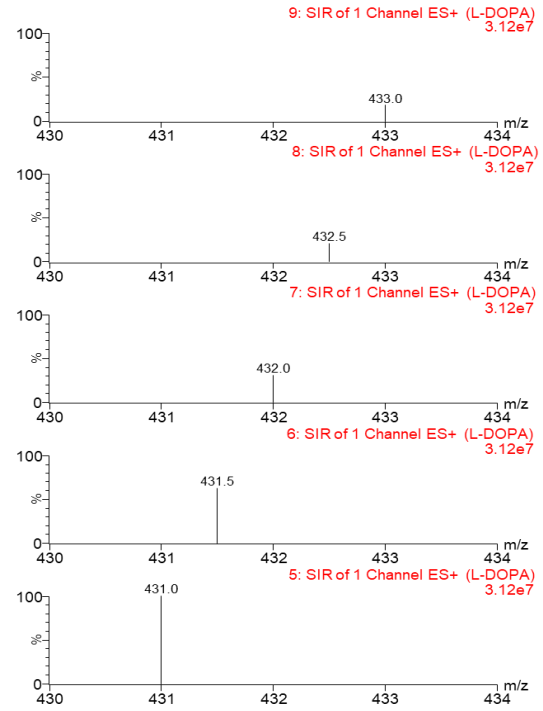
Table 3. MRM values for Dabsyl-Cl derivatization.

Neurotransmitter	DAB SIR Value	Quant Transition	CID Quant	Qual Transition	CID Qual
Dopamine	439.5	225.3	25	75.5	40
L-DOPA	485.0	225.3	25	75.6	45
Serotonin	464.5	224.2	25	120.0	40
Tyrosine	469.5	225.2	30	119.9	40
Tryptophan	492.5	224.1	20	120.0	40
Noradrenaline	457.5	225.3	20	75.6	45
Tyramine	425.5	120.0	30	169.0	25

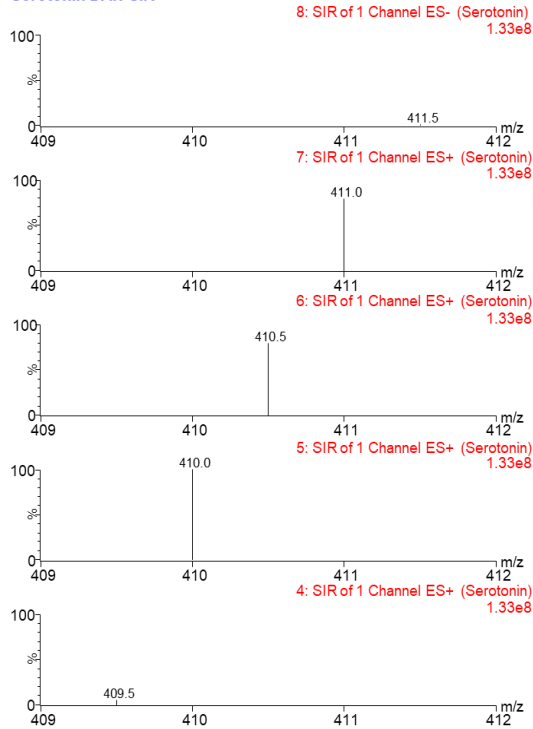
Dopamine DAN SIR



L-DOPA DAN SIR



Serotonin DAN SIR



Tyrosine DAN SIR

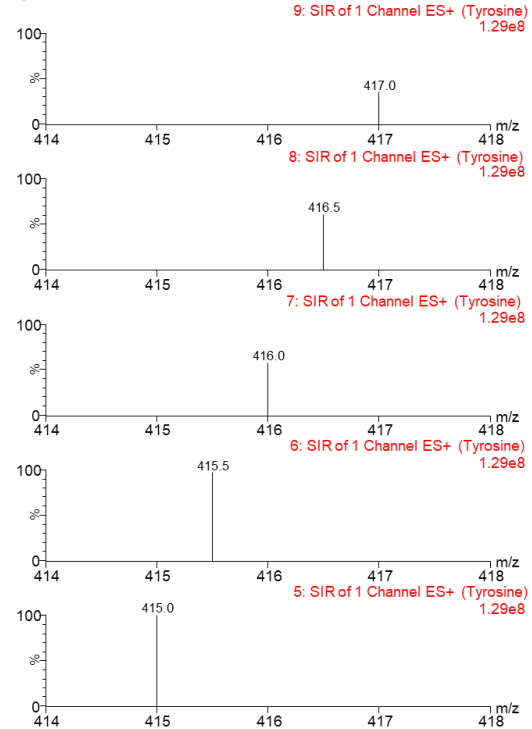
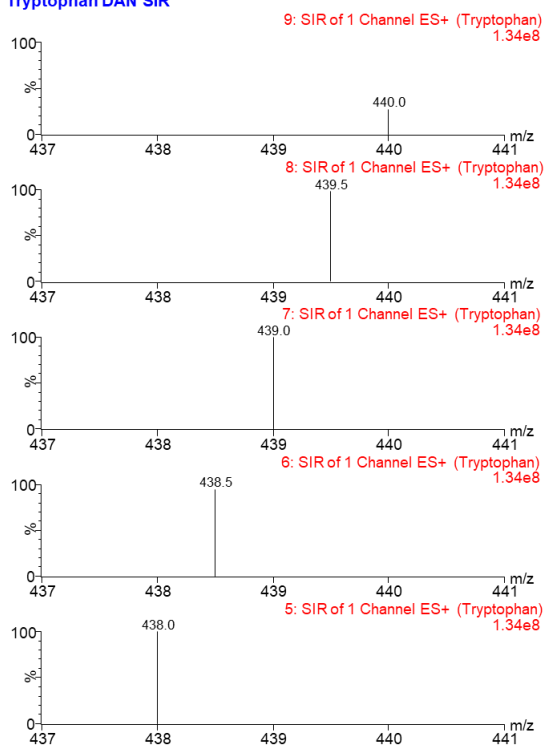
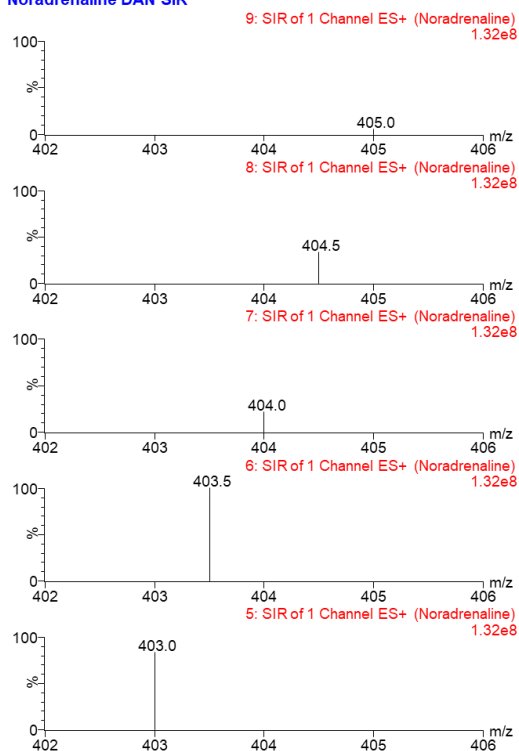


Figure 23. Dansyl SIR scans for dopamine, L-DOPA, serotonin, and tyrosine.

Tryptophan DAN SIR



Noradrenaline DAN SIR



Tyramine DAN SIR

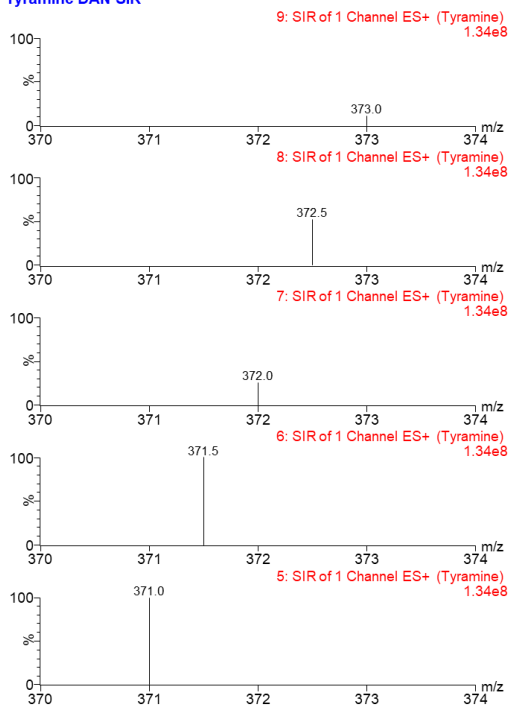
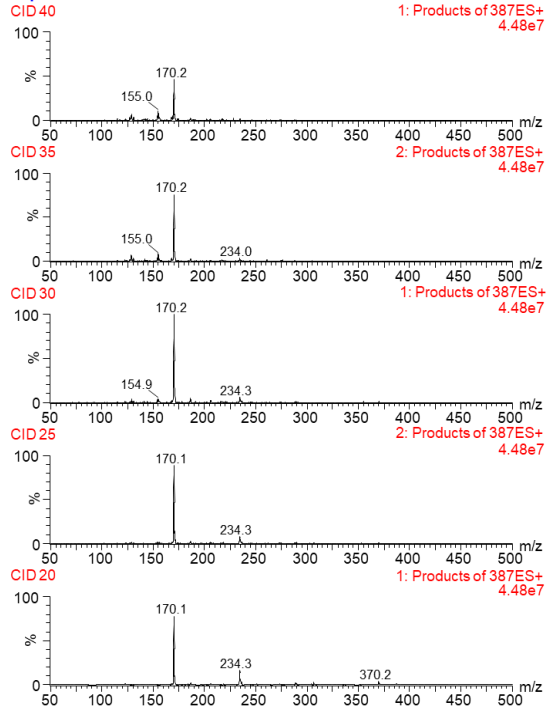
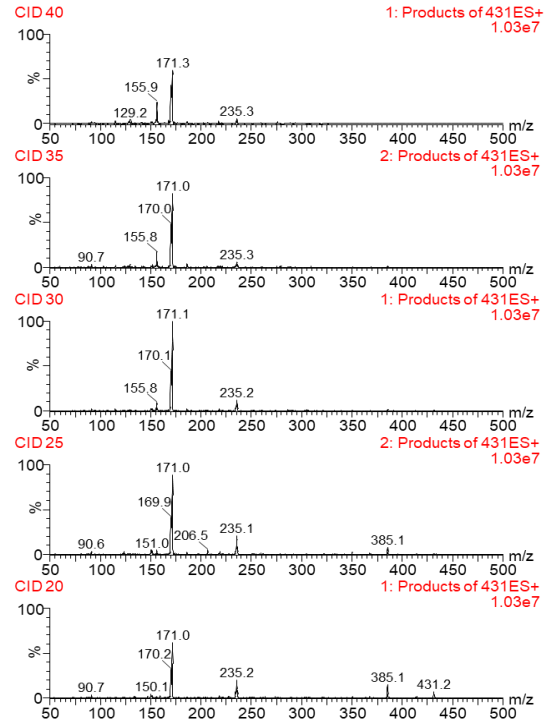


Figure 24. Dansyl SIR scans for tryptophan, noradrenaline, and tyramine.

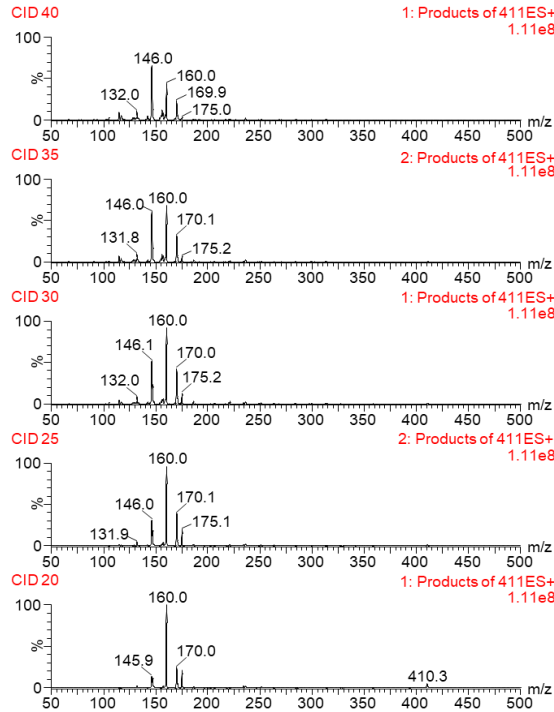
Dopamine DAN Product Ions



L-DOPA DAN Product Ions



Serotonin DAN Product Ions



Tyrosine DAN Product Ions

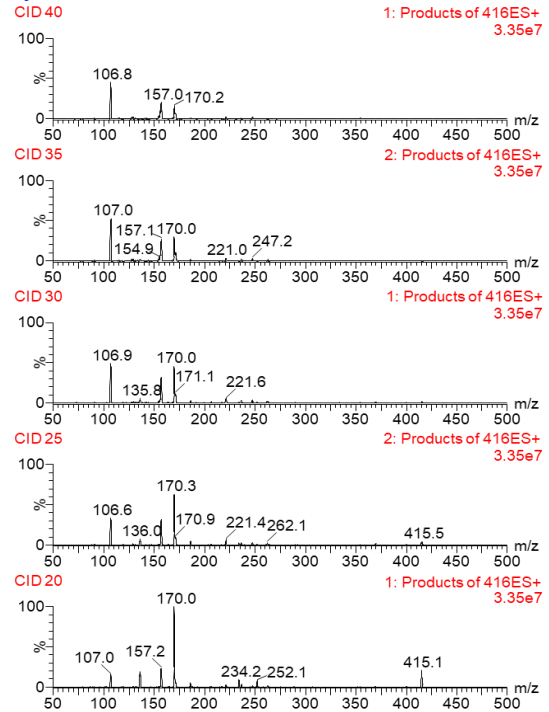
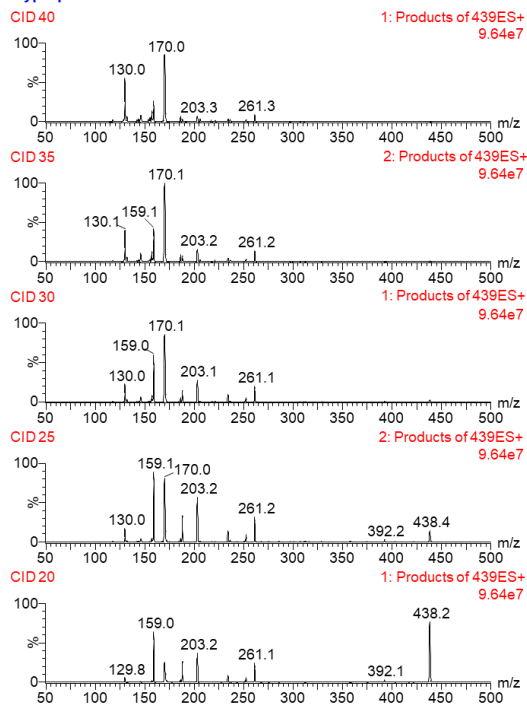
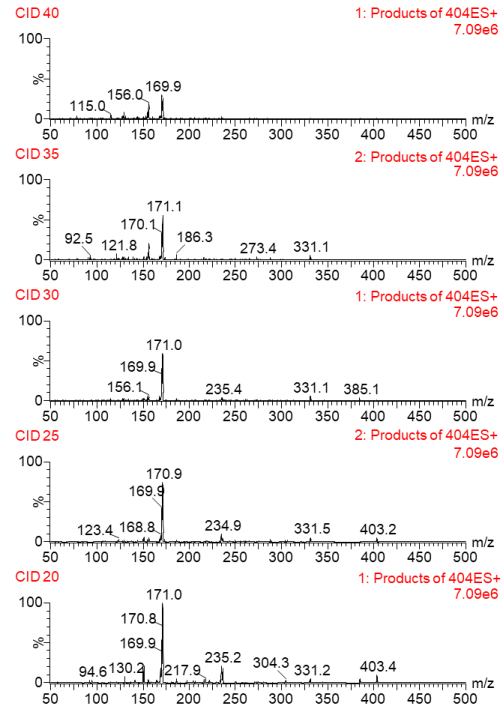


Figure 25. Dansyl Product Ion scans for dopamine, L-DOPA, serotonin, and tyrosine.

Tryptophan DAN Product Ions



Noradrenaline DAN Product Ions



Tyramine DAN Product Ions

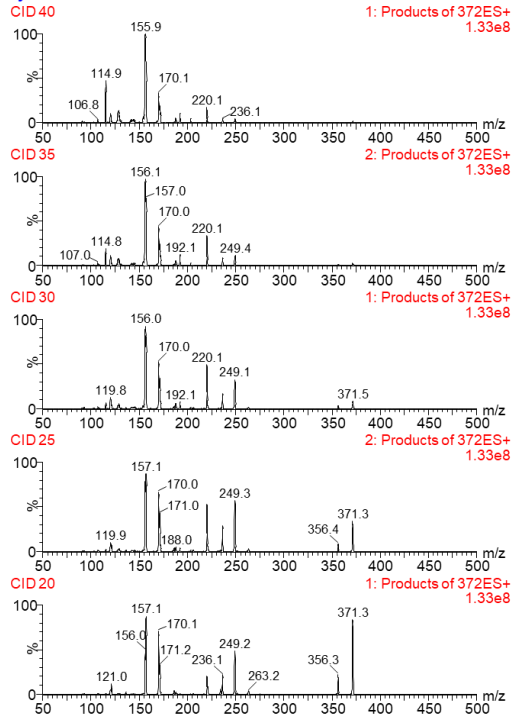


Figure 26. Dansyl Product Ion scans for tryptophan, noradrenaline, and tyramine.

Table 4. MRM values for Dansyl-Cl derivatization

Neurotransmitter	DAN SIR Value	Quant Transition	CID Quant	Qual Transition	CID Qual
Dopamine	387.0	170.1	25	156.0	40
L-DOPA	431.0	171.1	45	156.1	45
Serotonin	410.5	160.0	20	146.1	35
Tyrosine	415.5	170.0	20	106.8	30
Tryptophan	438.5	170.1	30	130.0	40
Noradrenaline	403.5	171.1	25	156.0	40
Tyramine	371.5	156.1	35	249.3	25

Chromatography Data

Once the MRM value were determined, a derivatization method was selected based on the chromatography. Initial tests were run for both Dabsyl-Cl and Dansyl-Cl at pH 8.5, 9.5, 10.5, and 14. Chromatograms for each neurotransmitter created with each method were compared to see which would give the best overall peak shapes and highest intensity. An example using tryptophan can be seen in Figure 27. Overall, it was decided that Dabsyl at pH 8.5 would be the best choice. Further testing could be done at pH 9.5 to compare the chromatographic results. After the method was decided, the neurotransmitter mix was derivatized accordingly and analyzed under the same conditions as the underivatized compound except that samples were prepared in acetone and ethyl acetate instead of water and methanol. Results of the 6x6 grid methods can be seen in Figure 28-33.

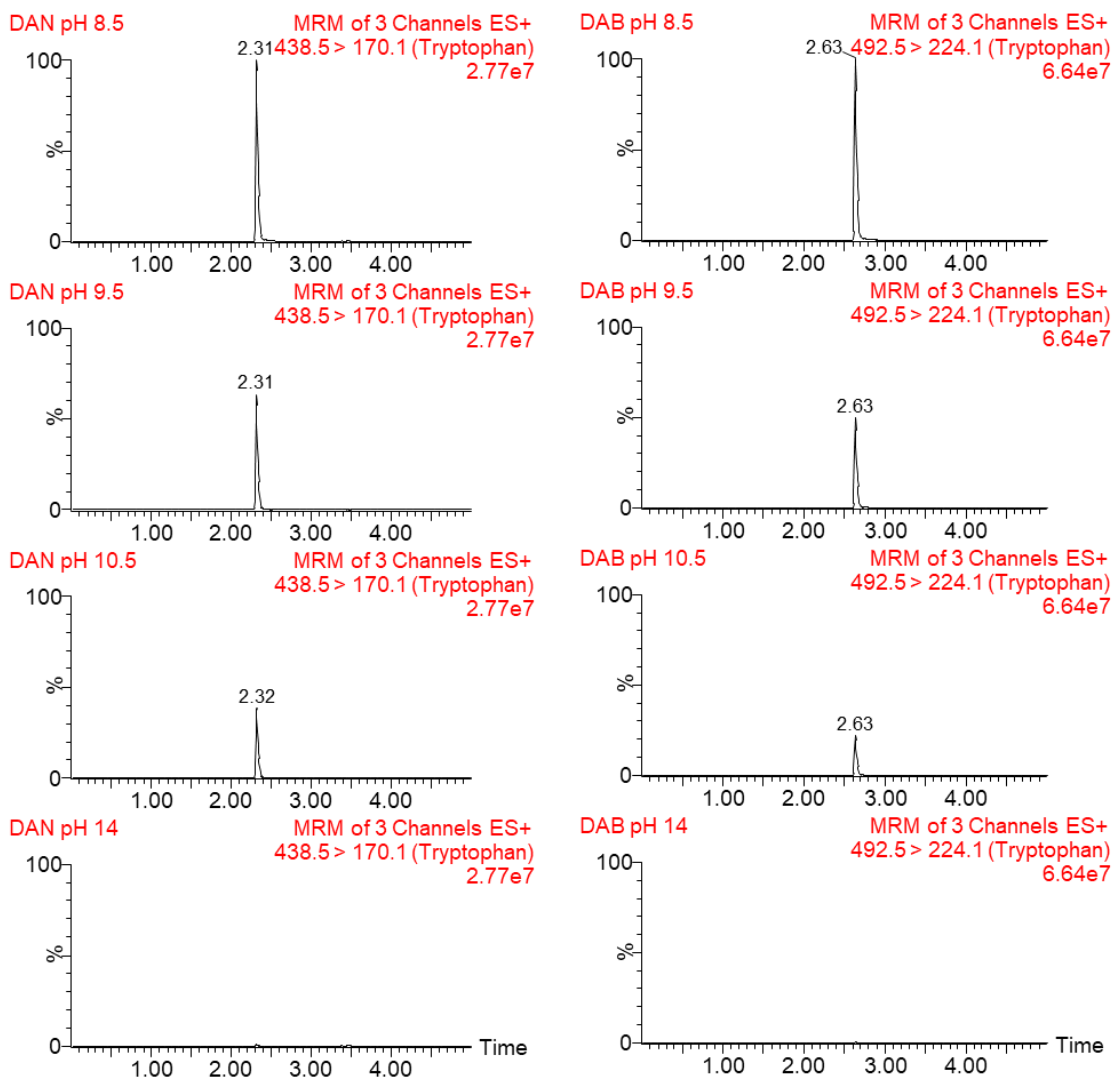


Figure 27. MRM chromatograms for tryptophan under DAN and DAB derivatizations at pH 8.5, 9.5, 10.5, and 14.

HSS T3 ACN pH 3 elution									
	1	2	3	4	5	6	7	8	9
Dopamine (ACN)	e5		e5	e4		e4	e5 MP		low sig
Dopamine (Ace)	e5		e5	e4		e4	e5 MP		low sig
Dopamine (EA)	e5		e5	e4		e4	e5 MP		
Noradrenaline (ACN)							low sig		low sig
Noradrenaline (Ace)							e5		low sig
Noradrenaline (EA)							low sig		low sig
Serotonin (ACN)	e5		broad	e5		e5	e5		e5 MP
Serotonin (Ace)	e5		broad	e5		e5	e5		e5 MP
Serotonin (EA)	e5		broad	e5		e5	e5		e5 MP
Tyrosine (ACN)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP
Tyrosine (Ace)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP
Tyrosine (EA)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP
L-DOPA (ACN)	e4 MP		broad	e4 MP		e4	low sig		low sig
L-DOPA (Ace)	e4 MP		broad	low sig		e4	low sig		low sig
L-DOPA (EA)	e4 MP		broad	e4 MP		e4	low sig		low sig
Tryptophan (ACN)	e6		e6	e6		e6	e7		tailing
Tryptophan (Ace)	e7		e6	e6		e6	e7		tailing
Tryptophan (EA)	e7		e6	e6		e6	e7		tailing
5-hydroxytryptophan (ACN)	e6		e5	e5		e5	e6		tailing
5-hydroxytryptophan (Ace)	e6		e5	e5		e5	e6		tailing
5-hydroxytryptophan(EA)	e6		e6	e5		e5	e6		tailing
Tyramine (ACN)	e6 MP		e6 MP	e6 MP		e6 MP	e7 MP		tailing
Tyramine (Ace)	e6 MP		e6 MP	e6 MP		e6 MP	e7 MP		tailing
Tyramine (EA)	e7 MP		e6 MP	e6 MP		e6 MP	e7 MP		tailing

HSS T3 MeOH pH 3elution									
	19	20	21	22	23	24	25	26	27
Dopamine (ACN)	e5		e4	low sig					
Dopamine (Ace)	e6		e5	e5					
Dopamine (EA)	e5		e5	low sig					
Noradrenaline (ACN)	low sig								
Noradrenaline (Ace)	low sig								
Noradrenaline (EA)	low sig								
Serotonin (ACN)	e6		tailing	e6					
Serotonin (Ace)	e6		tailing	e6					
Serotonin (EA)	e6		tailing	e6					
Tyrosine (ACN)	e6 MP		tailing	e6 MP					
Tyrosine (Ace)	e6 MP		tailing	e6 MP					
Tyrosine (EA)	e6 MP		tailing	e6 MP					
L-DOPA (ACN)	e5 MP		tailing	e5 MP					
L-DOPA (Ace)	e5 MP		tailing	e5 MP					
L-DOPA (EA)	e5 MP		tailing	e5 MP					
Tryptophan (ACN)	e7		tailing	e7					
Tryptophan (Ace)	e7		tailing	e7					
Tryptophan (EA)	e7		tailing	e7					
5-hydroxytryptophan (ACN)	e6		tailing	e6					
5-hydroxytryptophan (Ace)	e6		tailing	e6					
5-hydroxytryptophan(EA)	e6		tailing	e6					
Tyramine (ACN)	e7 MP		tailing	e6					
Tyramine (Ace)	e7 MP		tailing	e7 MP					
Tyramine (EA)	e7 MP		tailing	e7 MP					

Figure 28. 6x6 grid results HSS T3 elution derivatized.

BEH C18 ACN pH 3 elution									
	1	2	3	4	5	6	7	8	9
Dopamine (ACN)	e5		tailing	e5	e5	e5	e5		low sig
Dopamine (Ace)	e5		tailing	e5	e5	e5	e5 MP		low sig
Dopamine (EA)	e5		tailing	e5	e5	e5	e5 MP		low sig
Noradrenaline (ACN)	e5 MP		e5	low sig	e5	e5	low sig		e5 MP
Noradrenaline (Ace)	e5 MP		e5	e5 MP	e5	e5	e5 MP		e5 MP
Noradrenaline (EA)	e5 MP		e5	low sig	e5	e5	e5 MP		e5
Serotonin (ACN)	e6 MP		tailing	e5		tailing	e6		tailing
Serotonin (Ace)	e5 MP		tailing	e5		tailing	e5 MP		tailing
Serotonin (EA)	e5		tailing	e5		tailing	e5 MP		tailing
Tyrosine (ACN)	split		tailing	e6		tailing	e6		tailing
Tyrosine (Ace)	split		tailing	e6		tailing	e6		tailing
Tyrosine (EA)	e6 MP		tailing	e6		tailing	e6		tailing
L-DOPA (ACN)	e5 MP		tailing	low sig		tailing	e5		
L-DOPA (Ace)	e5 MP		low sig	e5 MP		low sig	e5		low sig
L-DOPA (EA)	e5		tailing	e5		tailing	e5		
Tryptophan (ACN)	e7		tailing	e6		tailing	e7		tailing
Tryptophan (Ace)	e7		tailing	e7		tailing	e7		tailing
Tryptophan (EA)	e7		tailing	e7		tailing	e7		tailing
5-hydroxytryptophan (ACN)	e6 MP		tailing	e6 MP		tailing	e6		tailing
5-hydroxytryptophan (Ace)	e6 MP		tailing	e6		tailing	e6		tailing
5-hydroxytryptophan(EA)	e6 MP		tailing	e6		tailing	e6		tailing
Tyramine (ACN)	e7 MP		tailing	e6 MP		tailing	e7 MP		tailing
Tyramine (Ace)	e6 MP		tailing	e7 MP		tailing	e7 MP		tailing
Tyramine (EA)	e7 MP		tailing	e7 MP		tailing	e7 MP		tailing

BEH C18 ACN pH 10 elution									
	10	11	12	13	14	15	16	17	18
Dopamine (ACN)	e5 MP		tailing	e5			e5 MP		
Dopamine (Ace)	e5 MP		tailing	e5 MP					e5 MP
Dopamine (EA)	e5 MP		tailing	e5					e5
Noradrenaline (ACN)	e5 MP		split	e5 MP		e6	e6 MP		tailing
Noradrenaline (Ace)	e5 MP		split	e6		e6	e6		tailing
Noradrenaline (EA)	e5 MP		split	e6 MP		e6	e6 MP		tailing
Serotonin (ACN)	e6 MP		tailing	e5 MP		tailing	e5 MP		tailing
Serotonin (Ace)	e6 MP		tailing	e5 MP		tailing	e5 MP		tailing
Serotonin (EA)	e6 MP		tailing	e5 MP		tailing	e5 MP		tailing
Tyrosine (ACN)	e7 MP		e7 MP	e7 MP		e7 MP	e7 MP		e7 MP
Tyrosine (Ace)	e7 MP		e7 MP	e7 MP		e7 MP	e7 MP		e7 MP
Tyrosine (EA)	e7 MP		e7 MP	e7 MP		e7 MP	e7 MP		e7 MP
L-DOPA (ACN)	e7 MP		e7 MP	e6		e6 MP	e6 MP		e7 MP
L-DOPA (Ace)	e6 MP		e6 MP	e6 MP		e5 MP	e5 MP		e5 MP
L-DOPA (EA)	e7 MP		e7 MP	e6		e6 MP	e6 MP		e6 MP
Tryptophan (ACN)	e8		e8	e8		e7	e8		e8
Tryptophan (Ace)	e8		e8	e8		e7	e7		e7
Tryptophan (EA)	e8		e8	e8		e8	e8		e8
5-hydroxytryptophan (ACN)	e7		e7 MP	e7		e7	e7		e7
5-hydroxytryptophan (Ace)	e7		e7 MP	e6 MP		e6 MP	e6 MP		
5-hydroxytryptophan(EA)	e7 MP		e7 MP	e7 MP		e7	e7		e7
Tyramine (ACN)	e8 MP		tailing	e7 MP		tailing	e7 MP		tailing
Tyramine (Ace)	e8 MP		tailing	e7 MP		tailing	e7 MP		tailing
Tyramine (EA)	e8 MP		tailing	e8 MP		tailing	e8 MP		tailing

Figure 29. 6x6 grid results BEH C18 ACN elution derivatized.

BEH C18 MeOH pH 3 elution											
	19	20	21		22	23	24		25	26	27
Dopamine (ACN)	e5		tailing		e5		e5		e5		
Dopamine (Ace)	e5		tailing		e5		e5		e5 MP		low sig
Dopamine (EA)	e5		tailing		e5		e5		e5 MP		low sig
Noradrenaline (ACN)	e5 MP		e5 MP		e5		e5		low sig		e5 MP
Noradrenaline (Ace)	e5 MP		e5		e5		e5		e5 MP		e5 MP
Noradrenaline (EA)	e5 MP		e5		e5		e5		e5		e5 MP
Serotonin (ACN)	e6 MP		tailing		e6		tailing		e6		tailing
Serotonin (Ace)	e5		tailing		e5		tailing		e5 MP		tailing
Serotonin (EA)	e5		tailing		e5		tailing		e5 MP		tailing
Tyrosine (ACN)	e6 MP		tailing		e6		tailing		e6		tailing
Tyrosine (Ace)	split		tailing		e6		tailing		e6 MP		tailing
Tyrosine (EA)	split		tailing		e6		tailing		e6 MP		tailing
L-DOPA (ACN)	e5 MP		tailing		e5		tailing		e5		
L-DOPA (Ace)	e5		low sig		e5 MP				e5 MP		
L-DOPA (EA)	e5		tailing		e5		tailing		e5		
Tryptophan (ACN)	e7		tailing		e7		tailing		e7		tailing
Tryptophan (Ace)	e7		tailing		e7		tailing		e7		tailing
Tryptophan (EA)	e7		tailing		e7		tailing		e7		tailing
5-hydroxytryptophan (ACN)	e6		tailing		e6		tailing		e6		tailing
5-hydroxytryptophan (Ace)	e6		tailing		e6		tailing		e6		tailing
5-hydroxytryptophan(EA)	e6		tailing		e6		tailing		e6		tailing
Tyramine (ACN)	e7		tailing		e6 MP		tailing		e7 MP		tailing
Tyramine (Ace)	e6		tailing		e7 MP		tailing		e7 MP		tailing
Tyramine (EA)	e7		tailing		e7 MP		tailing		e7 MP		tailing

BEH C18 MeOH pH 10 elution											
	28	29	30		31	32	33		34	35	36
Dopamine (ACN)	e5 MP										
Dopamine (Ace)	e5 MP										
Dopamine (EA)	e5 MP										
Noradrenaline (ACN)	e5 MP		low sig		fronting		low sig		low sig		low sig
Noradrenaline (Ace)	e5 MP		low sig		fronting		low sig		low sig		low sig
Noradrenaline (EA)	e5 MP		low sig		fronting		low sig		low sig		low sig
Serotonin (ACN)	e6 MP		tailing		tailing		low sig		tailing		low sig
Serotonin (Ace)	e6 MP		tailing		tailing		low sig		tailing		low sig
Serotonin (EA)	e6 MP		tailing		tailing		low sig		tailing		low sig
Tyrosine (ACN)	e7 MP		e5 MP		e5 MP		e5 MP		e5 MP		e5 MP
Tyrosine (Ace)	e7 MP		e5 MP		e5 MP		e5 MP		e5 MP		e5 MP
Tyrosine (EA)	e7 MP		e5 MP		e5 MP		e5 MP		e5 MP		e5 MP
L-DOPA (ACN)	e7 MP		low sig		fronting		low sig		e5		e5
L-DOPA (Ace)	e6 MP		low sig		low sig		low sig		low sig		low sig
L-DOPA (EA)	e7 MP		low sig		fronting		low sig		low sig		e5
Tryptophan (ACN)	e8		e6		e6		e5		e6		e6
Tryptophan (Ace)	e8		e6		e6		e5		e6		e5
Tryptophan (EA)	e8		e6		e6		e5		e6		e5
5-hydroxytryptophan (ACN)	e7		e5		e6		e5		e5		e5
5-hydroxytryptophan (Ace)	e7		e5		e6		e5		e5		e5
5-hydroxytryptophan(EA)	e7		e5		e6		e5		e5		e5
Tyramine (ACN)	e8 MP		tailing		tailing		tailing		tailing		tailing
Tyramine (Ace)	e8 MP		tailing		tailing		tailing		tailing		tailing
Tyramine (EA)	e8 MP		tailing		tailing		tailing		tailing		tailing

Figure 30. 6x6 grid results BEH C18 MeOH elution derivatized.

BEH C18 MeOH NH4F elution										
	19	20	21	22	23	24	25	26	27	
Dopamine (ACN)	e5 MP		low sig	low sig			tailing		low sig	
Dopamine (Ace)	e5		e5	tailing		tailing	tailing		low sig	
Dopamine (EA)	e5 MP		e5 MP	e5 MP					e5	
Noradrenaline (ACN)	e5 MP		e5			e5 MP	e5 MP		e5 MP	
Noradrenaline (Ace)	e5 MP		e5	low sig		e5 MP	e5 MP		e5 MP	
Noradrenaline (EA)	e5 MP		e5	e5 MP		e5 MP	e5 MP		e5 MP	
Serotonin (ACN)	tailing		tailing	tailing		tailing	tailing		tailing	
Serotonin (Ace)	tailing		tailing	tailing		tailing	tailing		tailing	
Serotonin (EA)	tailing		tailing	tailing		tailing	tailing		tailing	
Tyrosine (ACN)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP	
Tyrosine (Ace)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP	
Tyrosine (EA)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP	
L-DOPA (ACN)	tailing		e5	e5 MP		broad	broad		tailing	
L-DOPA (Ace)	tailing		e5 MP	e5 MP		e5 MP	tailing		e5 MP	
L-DOPA (EA)	tailing		e5	e5 MP		broad	broad		tailing	
Tryptophan (ACN)	e7		e7	e7		e7	e7 MP		e7	
Tryptophan (Ace)	e7		e7	e7		e7	e7 MP		e7	
Tryptophan (EA)	e7		e7	e7		e7	e7 MP		e7	
5-hydroxytryptophan (ACN)	e6 MP		e6 MP	tailing		e6 MP	tailing		tailing	
5-hydroxytryptophan (Ace)	e6 MP		e6 MP	tailing		e6 MP	tailing		tailing	
5-hydroxytryptophan(EA)	e6 MP		e6 MP	tailing		e6 MP	tailing		tailing	
Tyramine (ACN)	e7 MP		e7	e6		tailing	e7 MP		tailing	
Tyramine (Ace)	e7		e7 MP	e7 MP		tailing	e7 MP		tailing	
Tyramine (EA)	e7		e7 MP	e7 MP		tailing	e7 MP		tailing	

BEH C18 MeOH NH4Bicarbonate elution										
	28	29	30	31	32	33	34	35	36	
Dopamine (ACN)										
Dopamine (Ace)										
Dopamine (EA)										
Noradrenaline (ACN)	low sig			fronting		tailing	low sig		e4	
Noradrenaline (Ace)	low sig					tailing	low sig		e4	
Noradrenaline (EA)	e5			fronting		tailing	low sig		e4	
Serotonin (ACN)	tailing		tailing	tailing		low sig	tailing		low sig	
Serotonin (Ace)	tailing		tailing	tailing		low sig	tailing		low sig	
Serotonin (EA)	tailing		tailing	tailing		low sig	tailing		low sig	
Tyrosine (ACN)	e5 MP		e5 MP	e5 MP		e5 MP	e5 MP		e5 MP	
Tyrosine (Ace)	e5 MP		e5 MP	e5 MP		e5 MP	e5 MP		e5 MP	
Tyrosine (EA)	e5 MP		e5 MP	e5 MP		e5 MP	e5 MP		e5 MP	
L-DOPA (ACN)	e5		e4	shoulder		e4	e5		e5	
L-DOPA (Ace)	e5 MP		e4 MP	shoulder		e4	e4		e4 MP	
L-DOPA (EA)	e6		e4	shoulder		e4	e4		e5	
Tryptophan (ACN)	e6		e6	e6		e5	e6		e6	
Tryptophan (Ace)	e6		e6	e6		e5	e6		e5	
Tryptophan (EA)	e6		e6	e6		e5	e6		e5	
5-hydroxytryptophan (ACN)	e6		e5	e6		e5	e5		e5	
5-hydroxytryptophan (Ace)	e6		e5	e6		e5	e5 MP		e5	
5-hydroxytryptophan(EA)	e6		e5	e6		e5	e5 MP		e5	
Tyramine (ACN)	tailing		tailing	tailing		tailing	tailing		tailing	
Tyramine (Ace)	tailing		tailing	tailing		tailing	tailing		tailing	
Tyramine (EA)	tailing		tailing	tailing		tailing	tailing		tailing	

Figure 31. 6x6 grid results BEH C18 MeOH elution derivatized NH₄F and ammonium bicarbonate.

BEH Phenyl ACN pH 3 elution									
	1	2	3	4	5	6	7	8	9
Dopamine (ACN)	e5		e5 MP	e4		e4 MP	e5 MP		e4 MP
Dopamine (Ace)	e5		e5 MP	e5 MP		e5 MP	e5 MP		e4 MP
Dopamine (EA)	e5		e5 MP	e5 MP		e4 MP	e5 MP		e5 MP
Noradrenaline (ACN)	e4 MP		e4 MP	e4		e4 MP	e4 MP		e4 MP
Noradrenaline (Ace)	e4 MP		e5 MP	e4 MP		e4 MP	e4 MP		e4 MP
Noradrenaline (EA)	e4 MP		e5 MP	e4 MP		e4 MP	e4 MP		e4 MP
Serotonin (ACN)	e6 MP		e6	e6		e6	e7		e6
Serotonin (Ace)	e6 MP		e6 MP	e6		e6	e6 MP		e6 MP
Serotonin (EA)	e5 MP		e5 MP	e6		e6	e6 MP		e6 MP
Tyrosine (ACN)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP
Tyrosine (Ace)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP
Tyrosine (EA)	e6 MP		e6 MP	e6 MP		e6 MP	e6 MP		e6 MP
L-DOPA (ACN)	e5 MP		e5	e5		e5 MP	e5		e5
L-DOPA (Ace)	e5 MP		e5 MP	e4 MP		e4 MP	low sig		tailing
L-DOPA (EA)	e5 MP		e5	broad		e5 MP	e5		e5
Tryptophan (ACN)	e6		e7	e7		e7	e7		e7
Tryptophan (Ace)	e7		e7	e6		e6	e7		e7
Tryptophan (EA)	e7		e7	e7		e7	e7		e7
5-hydroxytryptophan (ACN)	e6		e6	e6 MP		e6 MP	e6		e6
5-hydroxytryptophan (Ace)	e6		e6	e6 MP		e6 MP	e6 MP		e6 MP
5-hydroxytryptophan(EA)	e6		e6	e6 MP		e6 MP	e6 MP		e6 MP
Tyramine (ACN)	e6 MP		e7 MP	e6		e6	e7 MP		e7
Tyramine (Ace)	e7 MP		e7 MP	e7		e7	e7 MP		e7
Tyramine (EA)	e7 MP		e7 MP	e7		e7	e7 MP		e7

BEH Phenyl ACN pH 10 elution									
	10	11	12	13	14	15	16	17	18
Dopamine (ACN)	e5 MP		e5 MP	low sig		e5 MP	e5 MP		e5 MP
Dopamine (Ace)	e5 MP		e5 MP	low sig		low sig	e5 MP		e5 MP
Dopamine (EA)	e5 MP		e5 MP	e5 MP		e5 MP	e6 MP		e6 MP
Noradrenaline (ACN)	e5 MP		e5 MP	e5 MP		e5 MP	e5 MP		e5 MP
Noradrenaline (Ace)	e5 MP		e5 MP	e5 MP		e5 MP	e5 MP		e5 MP
Noradrenaline (EA)	e5 MP		e5 MP	e5 MP		e5 MP	e6 MP		e6 MP
Serotonin (ACN)	e7		e7	e5 MP		e5 MP	e5 MP		e5 MP
Serotonin (Ace)	e6 MP		e6 MP	e5 MP		e5 MP	e5 MP		e5 MP
Serotonin (EA)	e6 MP		e6 MP	e5 MP		e5 MP	e5 MP		e5 MP
Tyrosine (ACN)	e7 MP		e7 MP	e6 MP		e7 MP	e7 MP		e7 MP
Tyrosine (Ace)	e7 MP		e7 MP	e7 MP		e7 MP	e7 MP		e7 MP
Tyrosine (EA)	e7 MP		e7 MP	e7 MP		e7 MP	e7 MP		e7 MP
L-DOPA (ACN)	e6 MP		e6 MP	tailing		e6 MP	e6 MP		e6
L-DOPA (Ace)	e6 MP		e6 MP	e5 MP		e5 MP	e5 MP		e5 MP
L-DOPA (EA)	e6 MP		e6 MP	tailing		e6 MP	e6 MP		e6
Tryptophan (ACN)	e8		e8	e7		e7	e7		e7
Tryptophan (Ace)	e8		e8	e7		e7	e7		e7
Tryptophan (EA)	e7		e7	e7		e7	e7		e7
5-hydroxytryptophan (ACN)	e7		e7	e6		e7	e7		e7
5-hydroxytryptophan (Ace)	e7		e7	e6 MP		e6 MP	e6 MP		e6 MP
5-hydroxytryptophan(EA)	e7		e7	e7 MP		e7 MP	e6 MP		e7 MP
Tyramine (ACN)	e7 MP		e7 MP	e7		e7 MP	e7 MP		e7 MP
Tyramine (Ace)	e7 MP		e7 MP	e7		e7 MP	e7 MP		e7 MP
Tyramine (EA)	e7 MP		e7 MP	e7		e7	e7 MP		e7 MP

Figure 32. 6x6 grid results BEH Phenyl ACN elution derivatized.

BEH Phenyl MeOH NH4F elution									
	19	20	21	22	23	24	25	26	27
Dopamine (ACN)	e6 MP		tailing	e5 MP		tailing	e5 MP		tailing
Dopamine (Ace)	e6 MP		tailing	e5 MP		tailing	e5 MP		tailing
Dopamine (EA)	e6 MP		tailing	e5 MP		tailing	e5 MP		tailing
Noradrenaline (ACN)	e5 MP		tailing	e5 MP		e5 MP	e5 MP		low sig
Noradrenaline (Ace)	e5 MP		tailing	e5 MP		e5 MP	e5 MP		low sig
Noradrenaline (EA)	e5 MP		tailing	e5 MP		e5 MP	e5 MP		low sig
Serotonin (ACN)	e7		tailing	e7		tailing	e7		tailing
Serotonin (Ace)	e6 MP		tailing	e6		tailing	e6 MP		tailing
Serotonin (EA)	e6 MP		tailing	e6		tailing	e6 MP		tailing
Tyrosine (ACN)	e7 MP		tailing	e7 MP		tailing	e6 MP		tailing
Tyrosine (Ace)	e7 MP		tailing	e7 MP		tailing	e7 MP		tailing
Tyrosine (EA)	e7 MP		tailing	e7 MP		tailing	e7 MP		tailing
L-DOPA (ACN)	e5 MP		tailing	e5 MP		tailing	e5 MP		
L-DOPA (Ace)	e5 MP		tailing	e5 MP		tailing	e5 MP		
L-DOPA (EA)	e5 MP		tailing	e5 MP		tailing	e5 MP		
Tryptophan (ACN)	e7		tailing	e7		tailing	e7		tailing
Tryptophan (Ace)	e7		tailing	e7			e7		tailing
Tryptophan (EA)	e8		tailing	e7			e7		tailing
5-hydroxytryptophan (ACN)	e7		tailing	e6 MP		tailing	e6		
5-hydroxytryptophan (Ace)	e7		tailing	e6 MP			e6 MP		
5-hydroxytryptophan(EA)	e7		tailing	e6 MP			e6 MP		
Tyramine (ACN)	e7 MP		tailing	e7		tailing	e7 MP		tailing
Tyramine (Ace)	e7 MP		tailing	e7		tailing	e7 MP		tailing
Tyramine (EA)	e7 MP		tailing	e7		tailing	e7 MP		tailing

BEH Phenyl MeOH NH4Bicarbonate elution									
	28	29	30	31	32	33	34	35	36
Dopamine (ACN)	e5 MP			low sig		low sig	low sig		broad
Dopamine (Ace)	e5 MP			low sig		e5 MP	e5 MP		broad
Dopamine (EA)	e5 MP			e5 MP		e5 MP	e5 MP		broad
Noradrenaline (ACN)	e5 MP		tailing			fronting	e5 MP		
Noradrenaline (Ace)	e5 MP		tailing	e5 MP		tailing	e5 MP		
Noradrenaline (EA)	e5 MP		tailing	e5 MP		tailing	e5 MP		
Serotonin (ACN)	e7		tailing	e6		tailing	tailing		tailing
Serotonin (Ace)	e6		tailing	e6		tailing	tailing		tailing
Serotonin (EA)	e6		tailing	e6		tailing	tailing		tailing
Tyrosine (ACN)	e7 MP		tailing	e7 MP		tailing	e7 MP		tailing
Tyrosine (Ace)	e7 MP		tailing	e7 MP		tailing	e7 MP		tailing
Tyrosine (EA)	e7 MP		tailing	e7 MP		tailing	e7 MP		tailing
L-DOPA (ACN)	e6 MP		tailing	e6 MP		tailing	e6 MP		tailing
L-DOPA (Ace)	e6 MP		tailing	e5 MP		tailing	e5 MP		
L-DOPA (EA)	e6 MP		tailing	tailing		tailing	e6 MP		tailing
Tryptophan (ACN)	e7		tailing	e7		shoulder	e7		tailing
Tryptophan (Ace)	e8		tailing	e7		shoulder	e7		tailing
Tryptophan (EA)	e8		tailing	e7		shoulder	e7		tailing
5-hydroxytryptophan (ACN)	e7		tailing	e7		shoulder	e7		tailing
5-hydroxytryptophan (Ace)	e7		tailing	e7		shoulder	e7 MP		tailing
5-hydroxytryptophan(EA)	e7		tailing	e7		shoulder	e7		tailing
Tyramine (ACN)	e7 MP		tailing	e7		tailing	e7 MP		tailing
Tyramine (Ace)	e8 MP		tailing	e7		tailing	e7 MP		tailing
Tyramine (EA)	e8 MP		tailing	e7 MP		tailing	e7 MP		tailing

Figure 33. 6x6 grid results BEH Phenyl MeOH elution derivatized NH₄F and ammonium bicarbonate.

The derivatized chromatography for each method showed significant improvement in intensity and the development of a gaussian peak shape. Most of the methods resulted in a green indication including some that were green for every sample. The HSS T3 column had all green boxes for method 7 and 19, both of which were under pH 3 elution (Figure 28). BEH C18 showed success for all under methods 4, 7, 10, 13, 22, 25, and 28 (Figure 29 and 30). The most improved column was the BEH Phenyl under ACN pH 3 and 10 which were completely successful with the exception of method 4 and 9 which had one yellow box and method 13 which had two (Figure 32). MeOH methods for the BEH Phenyl column showed success in methods 19, 22, 25, and 28 (Figure 33). Results for the chromatography show that the derivatization process was an effective means of altering the structure of the neurotransmitters in order to achieve cleaner and more intense spectra. If used for extraction later, the ideal method would be method 10 using the BEH Phenyl column in conjunction with a C8 trap column under ACN elution at pH 10 and loading at pH 10 (Figure 32). Each resulting spectra had decent intensity and a desirable gaussian peak shape which is far improved from the pre-derivatization stage.

CONCLUSIONS

The study examined neurotransmitters before and after derivatization to determine whether or not the derivatization process could provide an improvement in chromatography. Neurotransmitters are challenging compounds to analyze due to their sensitivity to pH and light as well as spontaneous oxidation, low availability, and multiple pKa values. Current methods of analysis are time consuming and require complex processes. With the use of derivatization and multidimensional liquid chromatography, the preparation takes only an hour to prepare and has a run time of ten minutes per injection. The resulting chromatography gives intense gaussian-shaped peaks with clean separation. The overall run time for each sample is significantly less than traditional HPLC setups and can use the same reagents at smaller quantities. Sample lists can be automated for overnight and weekend runs which can save time on the part of the analyst and maximizes the instrument productivity.

FUTURE DIRECTIONS

Further study can be done by applying the derivatization process to different matrices such as plasma and urine to see how the sample preparation process affects the results as well as examine matrix recovery and interferences. The method developed was intended to be used in with SPE as sample preparation, and a comparison can be made as to when to apply the derivatization process: before or after sample preparation. As mentioned above, more derivatization agents are available to be applied to amine functionality and future studies could examine their effectiveness for neurotransmitters. Additionally, the neurotransmitters included in the study only represent a small portion of

what is present in the body and further research could be done on other amines as well as other categories.

LIST OF JOURNAL ABBREVIATIONS

Anal Bioanal Chem	Analytical and Bioanalytical Chemistry
Anal Chem	Analytical Chemistry
Anal Chim Acta	Analytica Chimica Acta
Ann Clin Biochem	Analytical Investigation Standing Committee of the Association of Clinical Biochemistry
Chem Neuro	ACS Chemical Neuroscience
Int J Analytical Mass	International Journal of Analytical Mass Spectrometry and Chromatography
J Chrom A	Journal of Chromatography A
J Chrom B	Journal of Chromatography B
J Pharm Biomed Anal	Journal of Pharmaceutical and Biomedical Analysis

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