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I, Elisa A Nickum, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Chemistry.

It is entitled:

Analysis of Regulated Drugs Using Chromatographic and Spectrophotometric Techniques Coupled with Spectroscopy: An Orthogonal Approach to Protecting Public Health

Student's name: **Elisa A Nickum**

This work and its defense approved by:

Committee chair: Peng Zhang, Ph.D.

Committee member: Anna Gudmundsdottir, Ph.D.

Committee member: Laura Sagle, Ph.D.

UNIVERSITY OF Cincinnati

26808

Analysis of Regulated Drugs Using Chromatographic and Spectrophotometric Techniques Coupled with Spectroscopy: An Orthogonal Approach to Protecting Public Health

A dissertation submitted to the Graduate School of the University of Cincinnati

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in the Department of Chemistry
of the College of Arts and Sciences
by

Elisa A. Nickum
B.S. Ohio University

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Committee Chair: Peng Zhang, Ph.D.

Abstract of Dissertation

A considerable number of dietary supplements suspected of containing phosphodiesterase-5 (PDE-5) inhibitors and substituted phenethylamines have been analyzed by the U.S. Food and Drug Administration. Often these samples are found to contain the active pharmaceutical ingredients (API) such as sildenafil or phentermine, and in many cases, products contain multiple PDE-5 inhibitors or substituted phenethylamines. In an analytical setting, it is important to confirm the presence of any API with two or more independent methods, and this requirement can often put undo strain on a laboratory. The development and use of methods that inherently contain two unique identification techniques is preferred, and the creation and validation of three of those methods is outlined here. First, direct deposit Fourier transform infrared detection and mass spectrometric detection (GC/FT-IR/MS) is used to identify PDE-5 inhibitors. Generally, GC/MS is not generally used for this category of drugs due to low volatility; PDE-5 inhibitors often co-elute and can produce non-specific electron ionization fragmentation patterns. In contrast, FT-IR has been proven to be more selective for identifying PDE-5 inhibitors, but is generally not as sensitive as spectrometric techniques. However, it has been shown that each technique can compensate for the other, which allows a wider range of usability. Using this combined technique can save time and resources while still delivering a high level of certainty in identification by providing results from two scientifically uncorrelated techniques. Multiple reference standards were utilized for method validation, including determination of linearity, dynamic range, and limit of detection. Second, a single HPLC-UV method has been developed for the determination of PDE-5 inhibitors and related analogs in

pharmaceutical dosage forms and dietary supplement products. Using this protocol, 14 PDE-5 inhibitor compounds can be separated and determined in a single analysis. Multiple reference standards were utilized for method validation, including determination of linearity, dynamic range, injection precision, limits of detection and quantitation, accuracy and precision. It was also demonstrated that, in cases where a standard for a specific analog is not readily available, another reference standard can be used to approximate the level of analog present, based on similarities in their chemical structures and absorbance spectra. Third, an HPLC-UV method has been developed for the determination of substituted phenethylamines in pre-workout dietary supplements. The number of these products on the market has greatly increased in recent years, and the labeled ingredients did not seem to account for amazing energy and euphoria experienced by users. Using GC-MS analysis, methamphetamine-like compounds are often detected but can be difficult to identify; as standards are not always available for comparison. To characterize any new analog, it is necessary to separate it from the matrix using an acid-base extraction, followed by HPLC-UV fraction collection and characterization using HRAM-MS and nuclear magnetic resonance. Recently, these techniques were used to characterize N-ethyl-α-ethylphenethylamine in a powdered drink supplement. Using this HPLC-UV method, five substituted phenethylamines can be separated and determined in a single analysis. Multiple reference standards were utilized for method validation, including determination of linearity, dynamic range and injection precision.

Acknowledgements

I would like to thank my advisor, Dr. Peng Zhang for his counsel and encouragement throughout my time at UC. I would also like to recognize my committee, Dr. Laura Sagle, Dr. Anna Gudmundsdottir and the late Dr. Joseph Caruso for their guidance and constructive criticism, to push me beyond my analytical comfort zone into a well-rounded researcher. Thank you to the FDA Forensic Chemistry Center for supporting me as I balanced my time between the professional and academic worlds. My sincerest gratitude goes to my colleagues and friends Laura Ciolino Ph.D., Anna Donnell Ph.D., Traci Hanley Ph.D., Sara Kern Ph.D., Kevin Kubachka Ph.D., Adam Lanzarotta Ph.D., and Tony Wilson Ph.D., who have traveled this path before me and were willing to give me advice on the process. I would also like to thank the University of Cincinnati and the Department of Chemistry.

Furthermore, I would like to thank Dr. Margot Brandi, Sarah Voelker, Merrie Jackson, Valerie Toomey and Samuel Gratz, Ph.D. for their support, encouragement technical assistance and scientific contributions. Last but not least, I would like to thank my husband Randy for rides to school, quiet time to study and write, high-fives, editing, listening and always being willing to go on an adventure the evening before my yearly talk so I can test out AV equipment and practice my presentation at UC.

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Chapter 1 – Identification and Determination of Phosphodiesterase-5 Inhibitors using Gas Chromatography with Fourier Transform Infrared Detection and Mass Spectrometric Detection

1.1. Introduction

Synthetic phosphodiesterase type-5 (PDE-5) inhibitors including sildenafil, vardenafil, and tadalafil are being detected with increasing regularity in products that are labeled as "all natural" herbal supplements that offer "sexual performance enhancement" [1-11]. Forensic and regulatory agencies are becoming increasingly more skilled at detecting and identifying PDE-5 inhibitors in these products using various analytical techniques such as high performance liquid chromatography with ultraviolet detection (HPLC-UV) [4, 5, 8, 12, 13] or liquid chromatography with mass spectrometric detection LC-MS [1-6, 9, 11, 13-16]. HPLC-UV is capable of rudimentary identification, based on retention time as UV spectra, but is valued for accurate quantitation at a low laboratory cost and relative ease of use. LC-MS is often used for identification, given the unique mass spectrum of each PDE-5 inhibitor, but is less often used for quantification in screening methods. These two methods can be successfully used together for identification and determination, but doing so involves a larger investment of time and resources in the lab.

Additional techniques that have been used for analyzing PDE-5 inhibitors include electrochemistry [17], gas chromatography with mass spectrometric detection (GC-MS) [16, 18-21] and Fourier transform infrared spectroscopy (FT-IR) [22-26]. While widely available in most forensic labs, and considered a first-line technique for the analysis of many drugs, GC-MS is inhibited by a comparatively poor limit of detection due to the relatively low volatility of PDE-5 inhibitors, and restricted in identification because of a

lack of structurally significant ions present in the mass spectrum. Additionally, structurally related compounds such as isomers may not be easily identified by mass spectrometry.

In contrast, FT-IR produces rich and unique spectra which are ideal for identification, making it better at separating structurally related compounds and isomers. Also, samples can be analyzed quickly with minimal preparation and with minimal damage to the sample, even to the point of being non-destructive. However, FT-IR is not as sensitive in cases of low analyte concentration, or when multiple analytes are present, without additional extractive preparation [27].

For identification purposes, GC-MS and FT-IR are cheaper and easier than LC-MS to operate and maintain, involve less technical training to use, and both systems are capable of supporting library searches. However, when it is necessary to confirm any detected compounds with two or more orthogonal techniques, excess time and resources can often be wasted in an already overextended forensic laboratory. Combining these techniques into one, gas chromatography with direct deposit Fourier transform infrared detection and mass spectrometric detection (GC/FT-IR/MS), can save a laboratory from unnecessary analyses because GC/FT-IR/MS can produce a high level of certainty in identification by providing results from two scientifically uncorrelated techniques [10, 28-32].

For quantitative purposes, GC-MS and FT-IR may be at a disadvantage to HPLC-UV. Generally, GC-MS and FT-IR do not have the reproducibility, linear range, limit of detection (LOD) and limit of quantitation (LOQ) of HPLC-UV. Some of these shortcomings can be mitigated by using a derivatization process [7, 19, 21] to increase

the volatility of the compounds analyzed, and to adjust the collection parameters of both detectors.

1.2. Experimental

1.2.1. Materials

Tadalafil was purchased by Cayman Chemical (Ann Arbor, MI), sildenafil citrate was purchased from US Pharmacopeia Convention (Rockville, MD) and vardenafil dihydrochloride salt was purchased from Santa Cruz Biotechnology (Dallas, TX). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (St. Louis, MO). 18MΩ·cm deionized H_2O was generated using a Milli-Q system (Millipore, Billerica, MA). Disposable non-sterile luer lok syringes and 30 mm nylon syringe filters with 0.45 µm pore size were purchased from Fisher Scientific.

1.2.2. Standard Preparation

Stock standards sildenafil, tadalafil and vardenafil (Figure 1.1) were prepared at approximately 1 mg per mL in methanol or acetonitrile. The solutions were vortexed and sonicated to dissolve. A mixed standard, of approximately equal concentrations sildenafil, tadalafil and vardenafil was prepared by combining equal amounts of each stock standard.

Standards were derivatized by evaporating aliquots of standard to dryness in an autosampler vial, at 90°C under a stream of nitrogen. Then, 200 µl pyridine and 200 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added to the vial before capping tightly and incubating for 90 minutes at 90°C. For ease, the entire reaction can be performed in the original autosampler vial and then directly analyzed.

Analysis of the resulting solutions was conducted using a fully integrated GC/FT-IR/MS instrument.

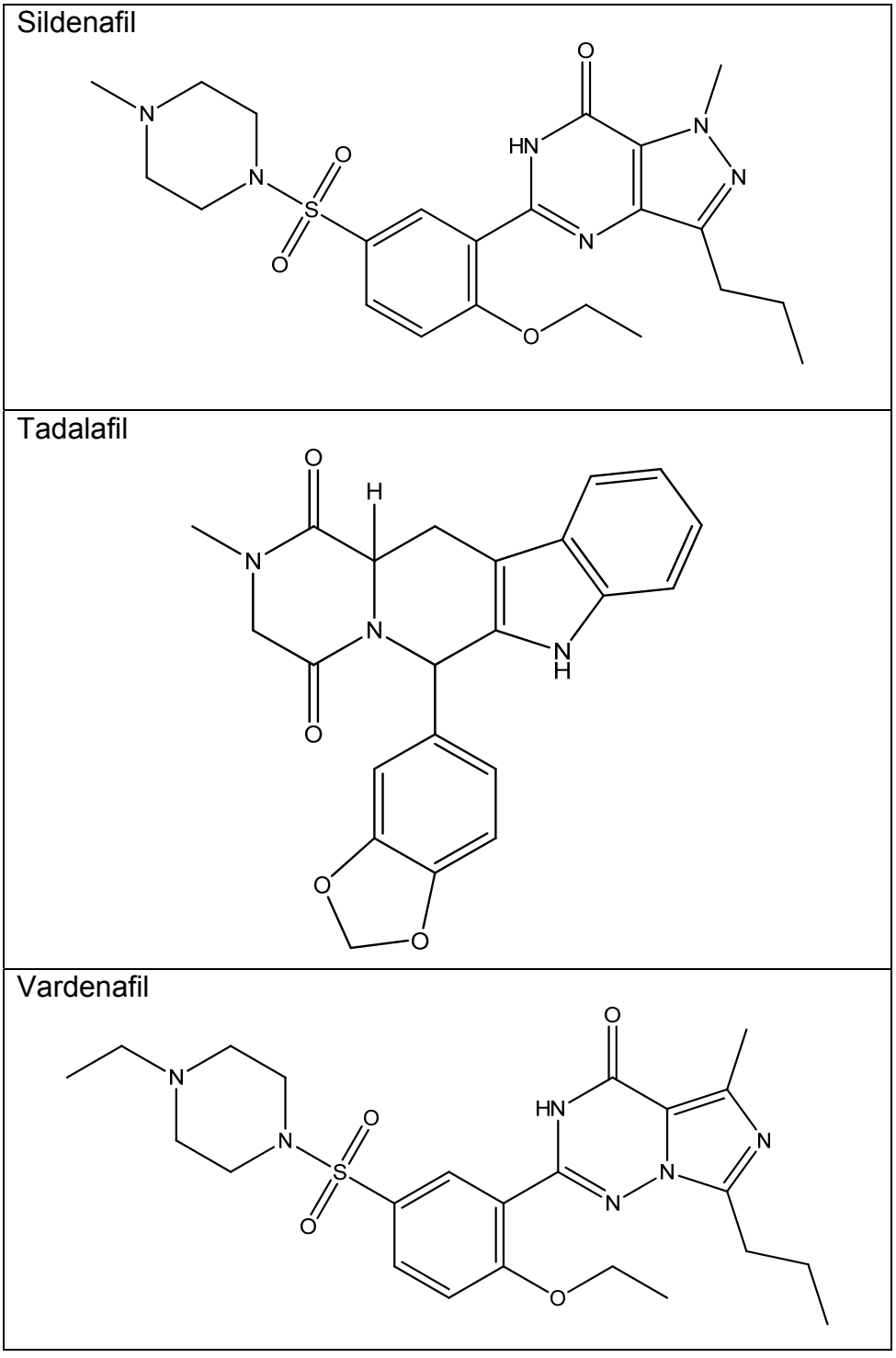

Figure 1.1. Structures of sildenafil, tadalafil and vardenafil

1.2.3. Gas Chromatography

Chromatography was conducted using an Agilent 7890 B Series GC outfitted with a G4567A Series autosampler and a Zebron ZB-5HT Inferno column from Phenomenex, (Torrance, CA) consisting of 5% phenyl – 95% dimethylpolysiloxane with a length of 30 m (5 m guard), internal diameter of 0.25 mm and a film thickness of 0.25 µm. Helium carrier gas was employed in constant flow mode using a flow rate of 2 mL/min. Injections were performed in splitless mode with an injection volume of 1.0 µL and an injector temperature of 300°C. The method included a starting temperature of 200°C with a ramp rate of 15°C/min to 350°C and the temperature was held for 10.0 min, for a total run time of 20 min. The terminus of the column was inserted into an inert capillary tee that splits approximately ¾ of the GC effluent to a transfer line connected to the IR interface and approximately ¼ of the GC effluent to a transfer line connected to the mass spec interface. The transfer line temperatures from the GC to the mass selective detector and from the GC to the infrared detector were 280°C and 300°C, respectively.

1.2.4. Infrared Detection

Infrared detection was accomplished using a Dani Instruments DiscovIR FT-IR spectrometer. The terminus of the transfer line from the GC was inserted into the IR interface and positioned directly above the ZnSe disk. FT-IR spectral data were collected using a 100 µm × 100 µm MCT detector, 4000–700 cm^{-1} spectral range, 4 cm^{-1} resolution, 3 mm/min disk speed, 4.0 min solvent delay, 19 min end time, 300°C restrictor temperature, 300°C oven temperature, 35°C dewar cap temperature and

−40°C disk temperature. Instrument operations and data analysis were conducted using workbooks designed in Grams software version 9.2 by Dani Instruments.

1.2.5. Mass Spectrometry

Mass spectrometric detection was performed using an Agilent 5977A series mass selective detector. The terminus of the second transfer line from the GC was inserted into the MS and positioned directly in front of the electron ionization source. Mass spectral data were collected from 30 to 700 amu using full scan mode, 3.80 min solvent delay, threshold of 150, sampling of 2, quadrupole temperature of 150°C and source temperature of 230°C. Instrument control and data collection for both the GC and MS were controlled by Agilent MSD ChemStation software version F.01.03.2357. Spectra were compared to the Wiley Registry of Mass Spectral Data, 10^{th} Edition and NIST11 Mass Spectral Library from the National Institute of Standards and Technology (Gaithersburg, MD).

1.3. Results and Discussion

1.3.1. GC/MS

Underivatized analysis of tadalafil, sildenafil and vardenafil yielded peaks at retention times of 12.4/12.6, 13.3 and 14 minutes, respectively (Figure 1.2). The two peaks related to tadalafil are likely due to the presence of stereoisomers. Both tadalafil peaks exhibited fragment ions at m/z 389, 262, 233, 204 and 169, which indicate a similar structure. The intensity of the molecular ion at m/z 389 is significant, suggesting stability of the intact molecule. The presence of additional ions is described in Figure 1.3. The fragmentation pattern of sildenafil does not produce many structurally

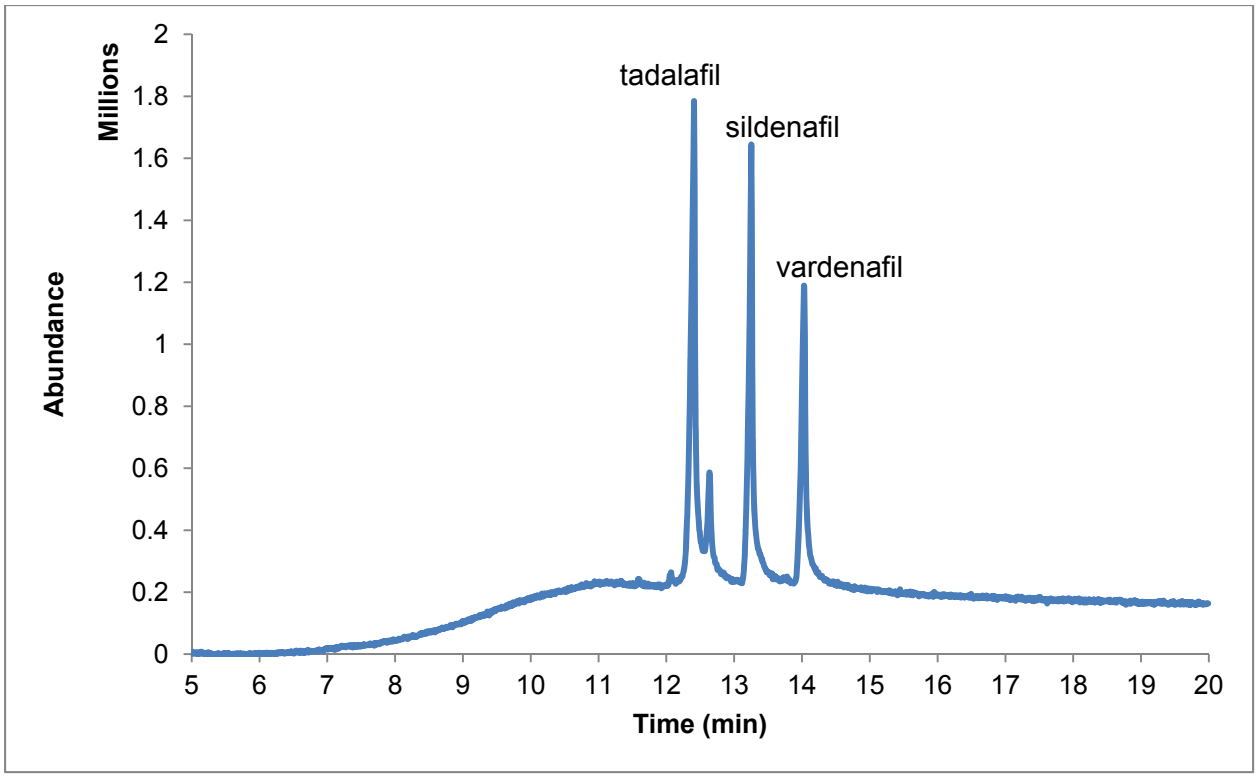

Figure 1.2. Chromatogram for the underivatized GC/MS analysis of tadalafil, sildenafil and vardenafil. Concentration values for the free base averaged 0.3 mg/mL.

significant ions of great abundance. The molecular ion at m/z 474 is only visible at higher concentrations, and the largest ion at m/z 99 is not highly significant. The same can be said for vardenafil, which has a molecular ion at m/z 488 and a fragmentation pattern similar to sildenafil (Figure 1.3).

Derivatized analysis of tadalafil, sildenafil and vardenafil yielded peaks at retention times of 11.6, 11.7 and 12 minutes, respectively. Worth noting, the response (graphed in Figure 1.4 as abundance) of the derivatized compounds at a concentration of 85 µg/mL is approximately twice as high when compared to analyzing the underivatized compounds at a concentration of 0.3 mg/mL. This shows a significant

Figure 1.3a: Tadalafil (Peak 1)

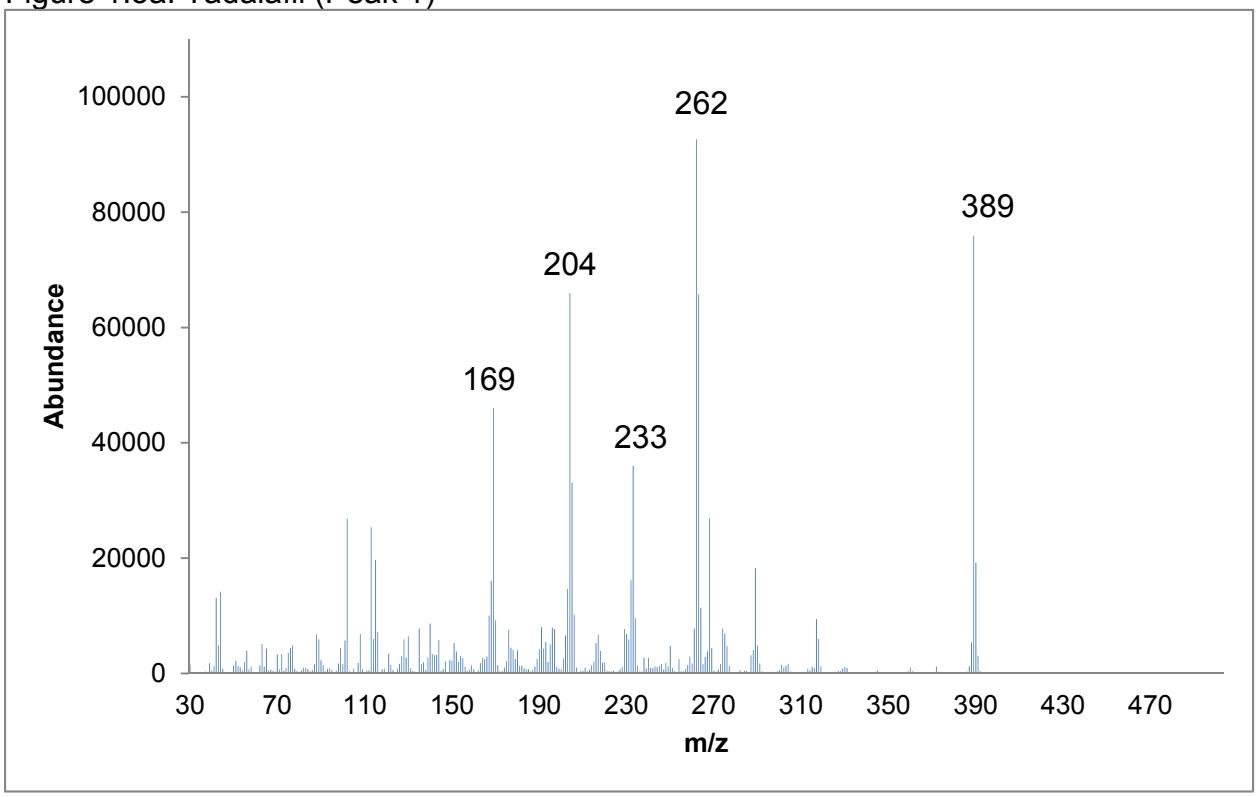

Figure 1.3b: Tadalafil (Peak 2)

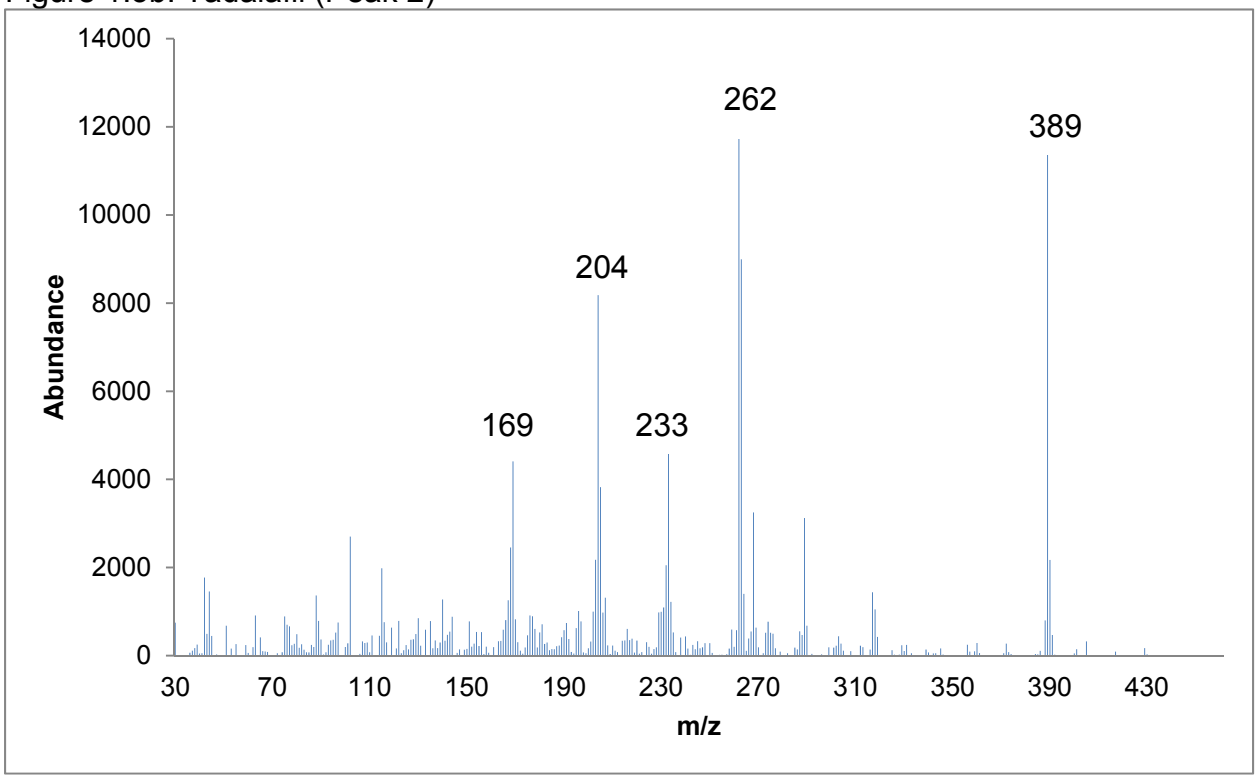

Figure 1.3c: Sildenafil

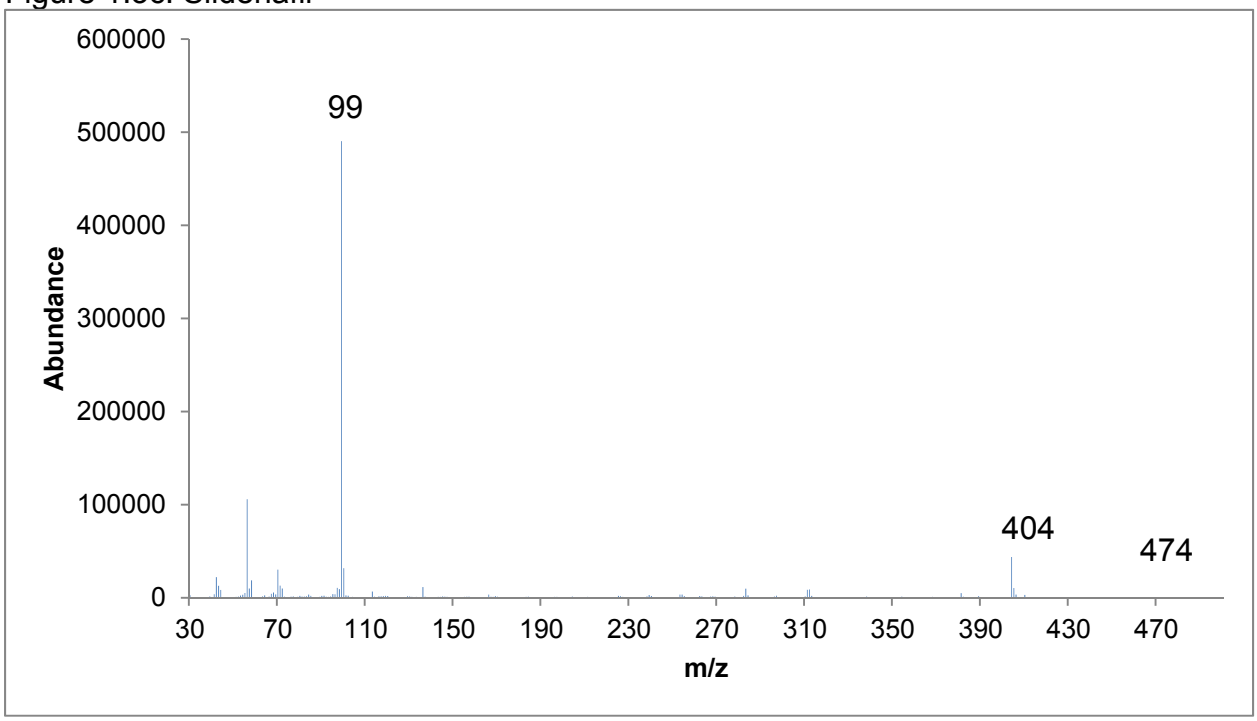

Figure 1.3d: Vardenafil

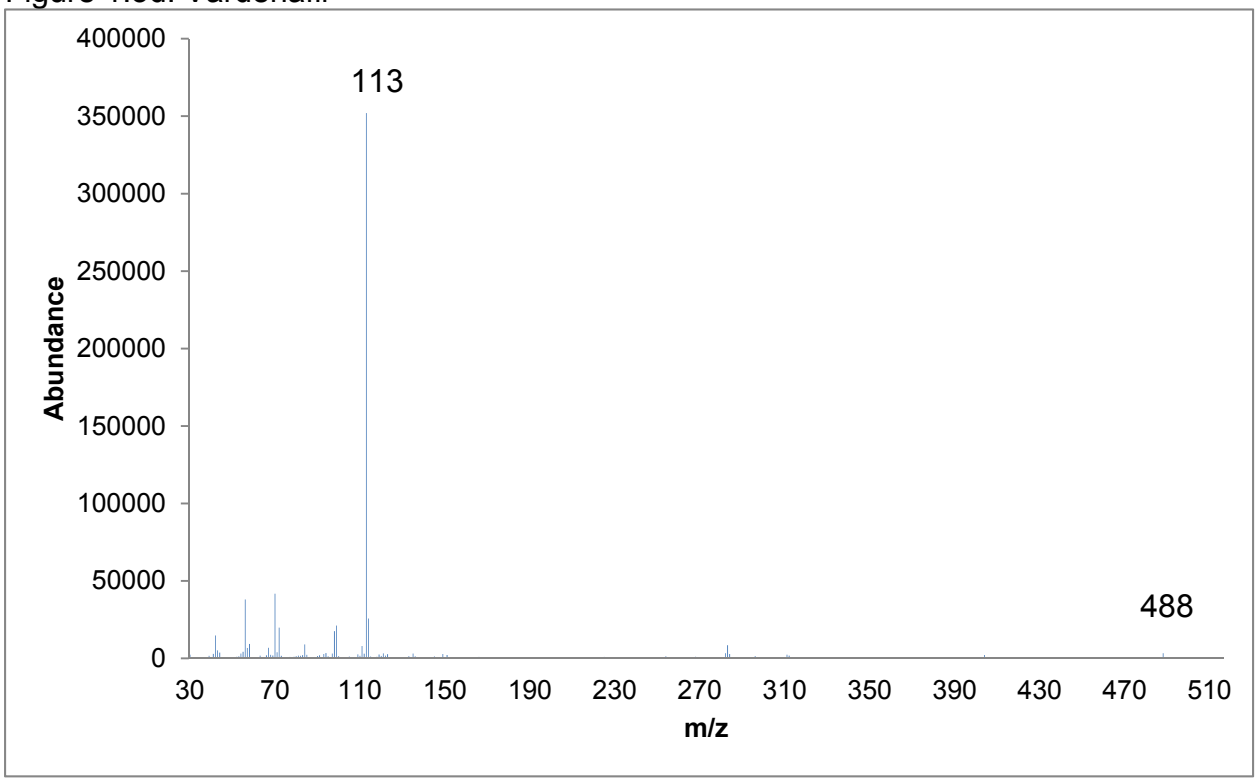

Figure 1.3. Mass spectral data for the underivatized analysis of tadalafil, sildenafil and vardenafil.

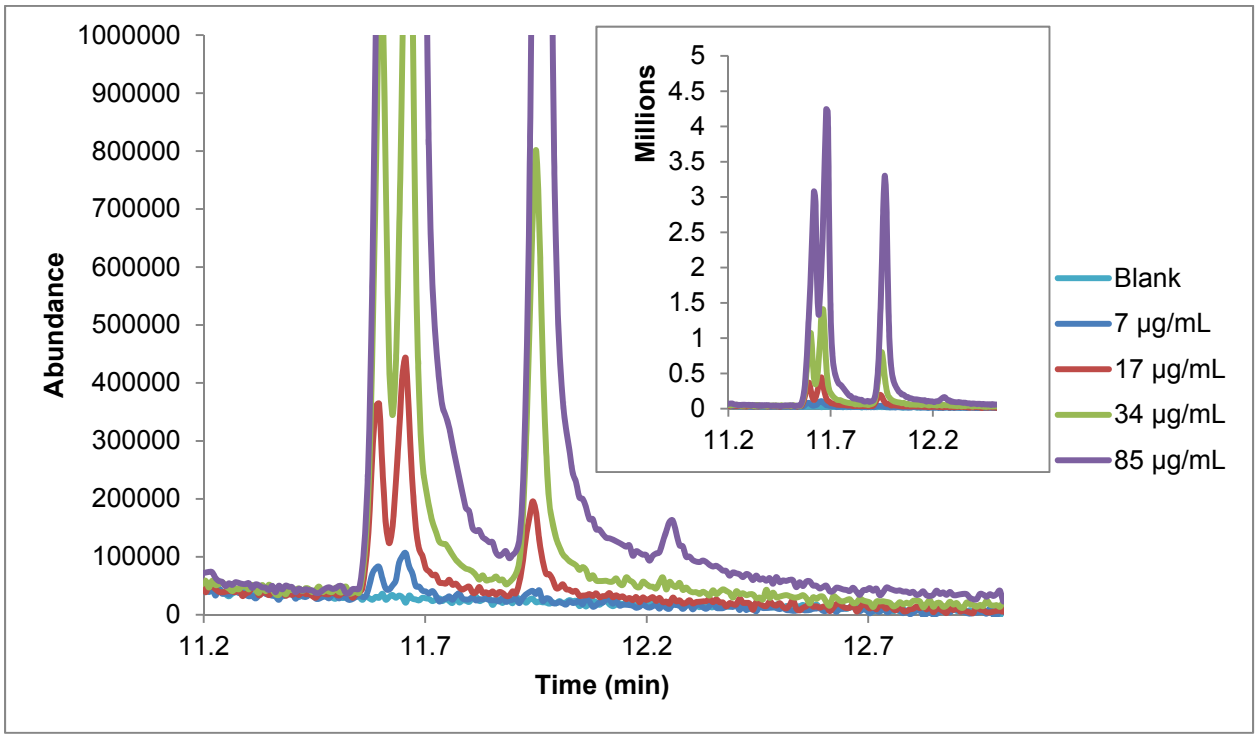

Figure 1.4. Overlay of chromatograms for the derivatized GC/MS analysis of mixtures of tadalafil, sildenafil and vardenafil. Concentration values for the free base ranged from 7 to 85 µg/mL.

increase in volatility of the derivatized compounds. Here, the tadalafil is present as only one peak, with the increased volatility of the compound producing a more chromatographically ideal response. The sildenafil peak has a very similar retention time to tadalafil, and the two peaks are not completely resolved. Similar to the underivatized analysis, the tadalafil peak exhibits a richer fragmentation pattern, with fragment ions at m/z 461, 340 and 241 (Figure 1.5). The ion with m/z 73 is present due to the trimethylsilyl derivatizing agent. The fragmentation pattern of sildenafil exhibited fragment ions at m/z 546, 476 and 99, but does not produce many structurally significant ions. As before, the molecular ion at m/z 546 is only visible at higher

concentrations, and the largest fragment ion at m/z 99 does not provide any significant structural information compared to the underivatized analysis. Vardenafil has a molecular ion at m/z 560 and the fragment ion at m/z 113 does not provide significant structural information.

Figure 1.5a: Tadalafil

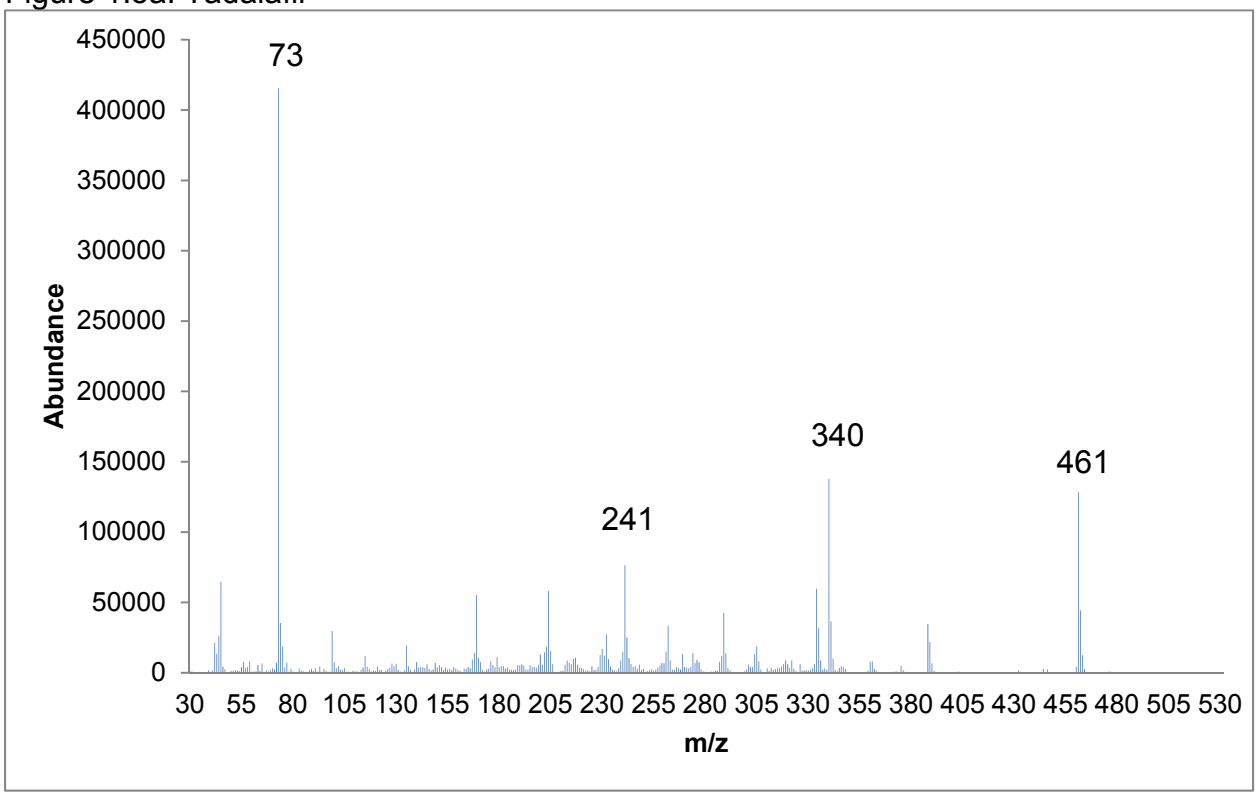

Figure 1.5b: Sildenafil

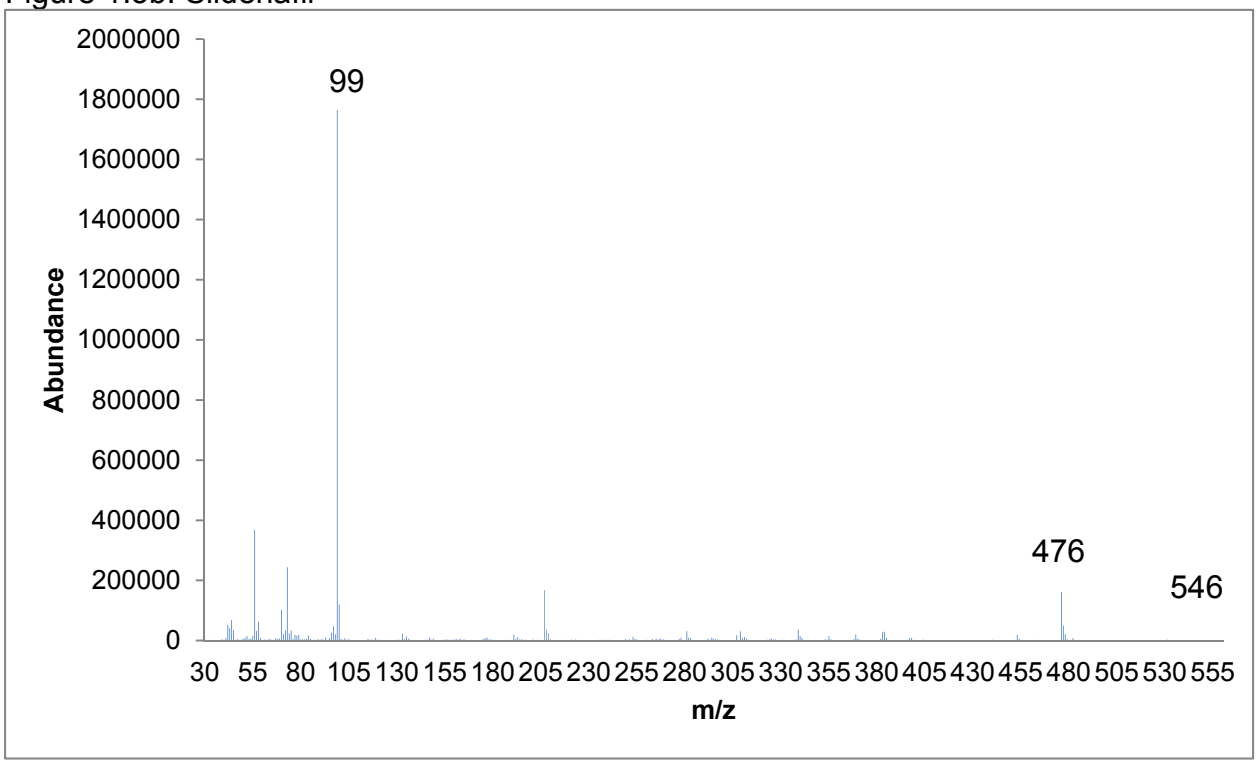

Figure 1.5c: Vardenafil

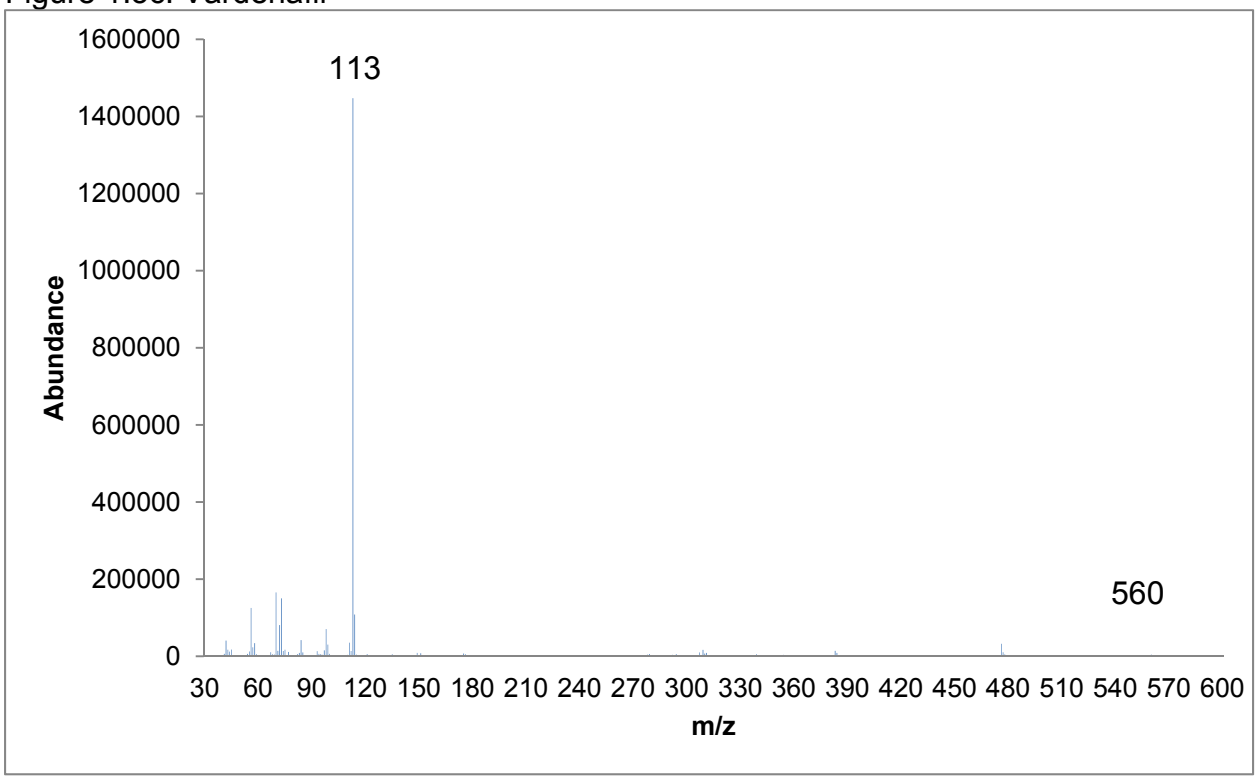

Figure 1.5. Mass spectral data for the derivatized analysis of tadalafil, sildenafil and vardenafil.

1.3.2. FT-IR

The retention times of underivatized tadalafil, sildenafil and vardenafil are 12.6/12.8, 13.6 and 14.4 minutes, respectively (Figure 1.6). The two peaks related to tadalafil are likely due to stereoisomers since their infrared spectra exhibited all of the same major infrared absorption bands with only slight differences in the region around 1450 cm^{-1}. Although each spectrum exhibited similarities, they could each be easily differentiated based on band shifting as well as unique bands in Figure 1.7.

When derivatized using TMS, tadalafil and sildenafil co-eluted at 11.9 minutes and vardenafil eluted at 12.2 minutes (Figure 1.8). Worth noting, the IR disc speed was slowed from 3 mm/min to 10 mm/min in an effort to increase resolution. While reducing the speed did improve peak separation, sensitivity was lost and it was also determined that the disc speed was not consistent enough to yield reproducible retention time data, given the significant shift in retention time between the 85 µg/mL and 34 µg/mL solutions. Compared to the underivatized spectra, each derivatized spectrum (Figure 1.9) exhibited an additional Si-CH_3 rocking absorption around 845 cm^{-1} and no N-H stretching absorption around 3330 cm^{-1}. The derivatized spectra for sildenafil and vardenafil also contain significantly suppressed secondary amide C=O stretching absorptions around 1705 cm^{-1} compared to those observed in the underivatized spectra. These differences between the underivatized and derivatized spectra confirm that that silylation occurred at the secondary amide position of the sildenafil and vardenafil analogs and at the secondary amine position of tadalafil.

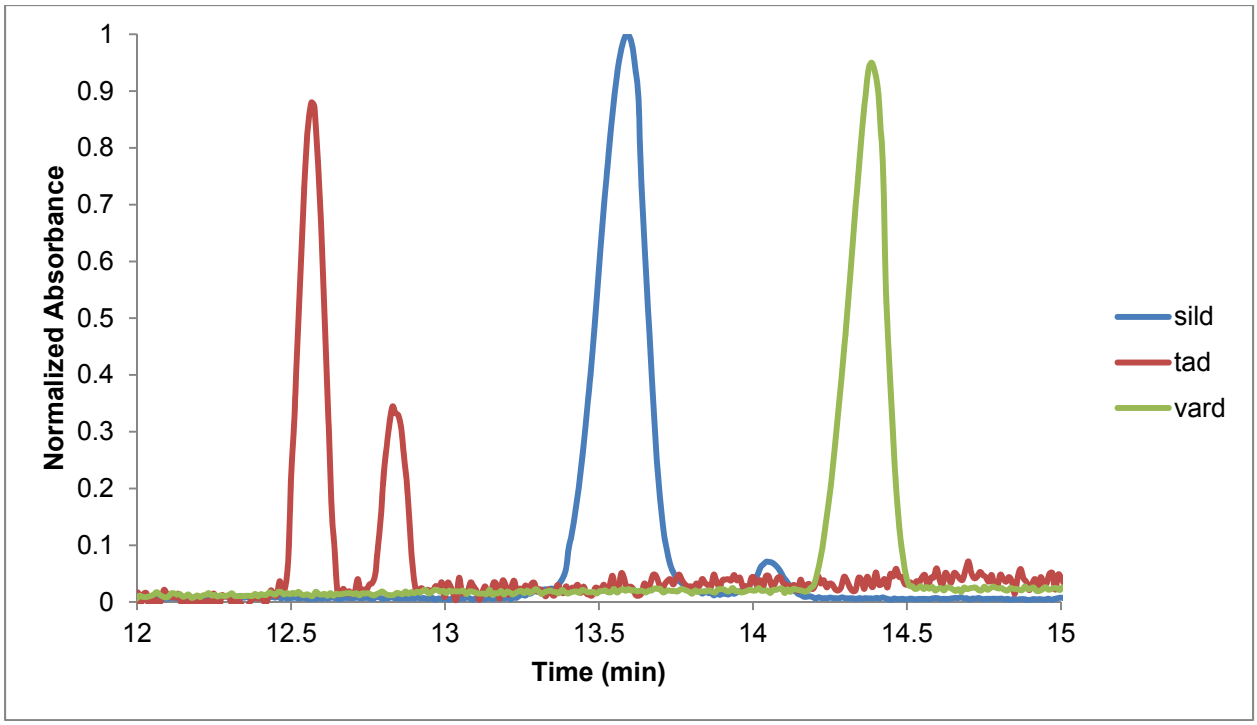

Figure 1.6. Overlay of chromatograms for the underivatized FT-IR analysis of tadalafil, sildenafil and vardenafil. Concentration values for the free base averaged 0.3 mg/mL.

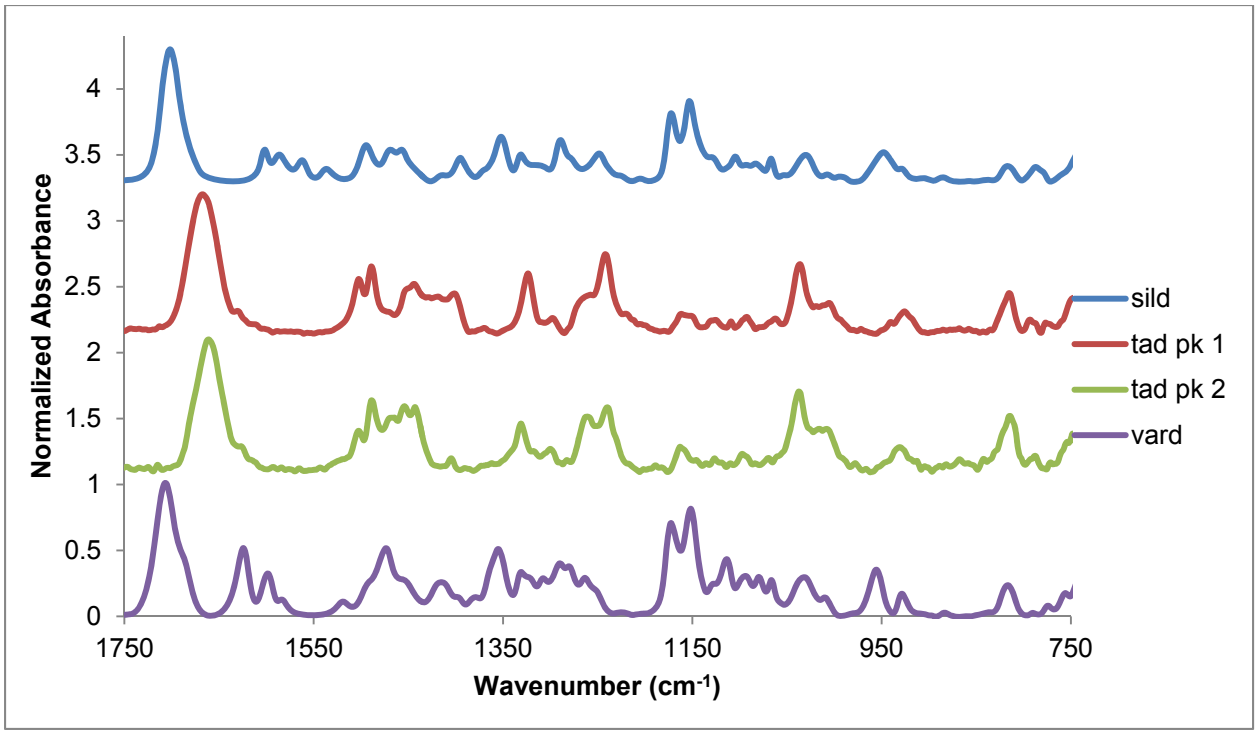

Figure 1.7. IR spectra for underivatized analysis of tadalafil, sildenafil and vardenafil.

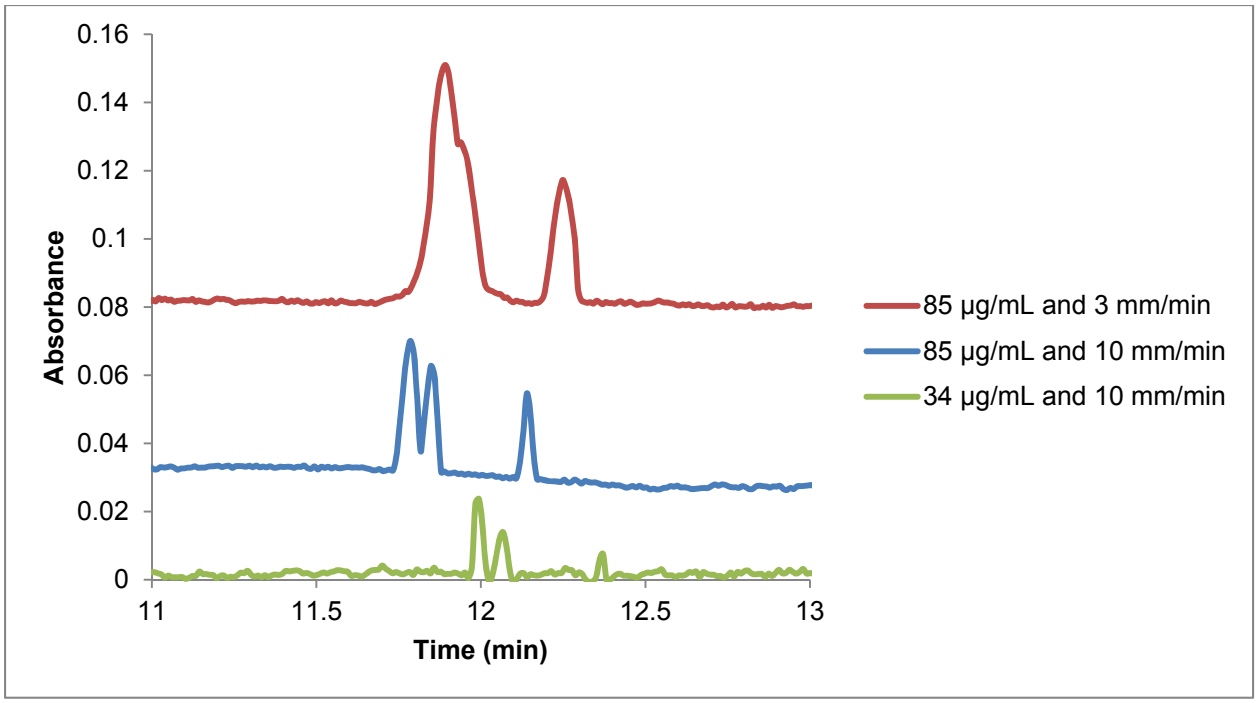

Figure 1.8. Overlay of chromatograms for the derivatized FT-IR analysis of mixtures of tadalafil, sildenafil and vardenafil at concentrations of 34 and 85 µg/mL and disc speeds of 3 and 10 mm/min.

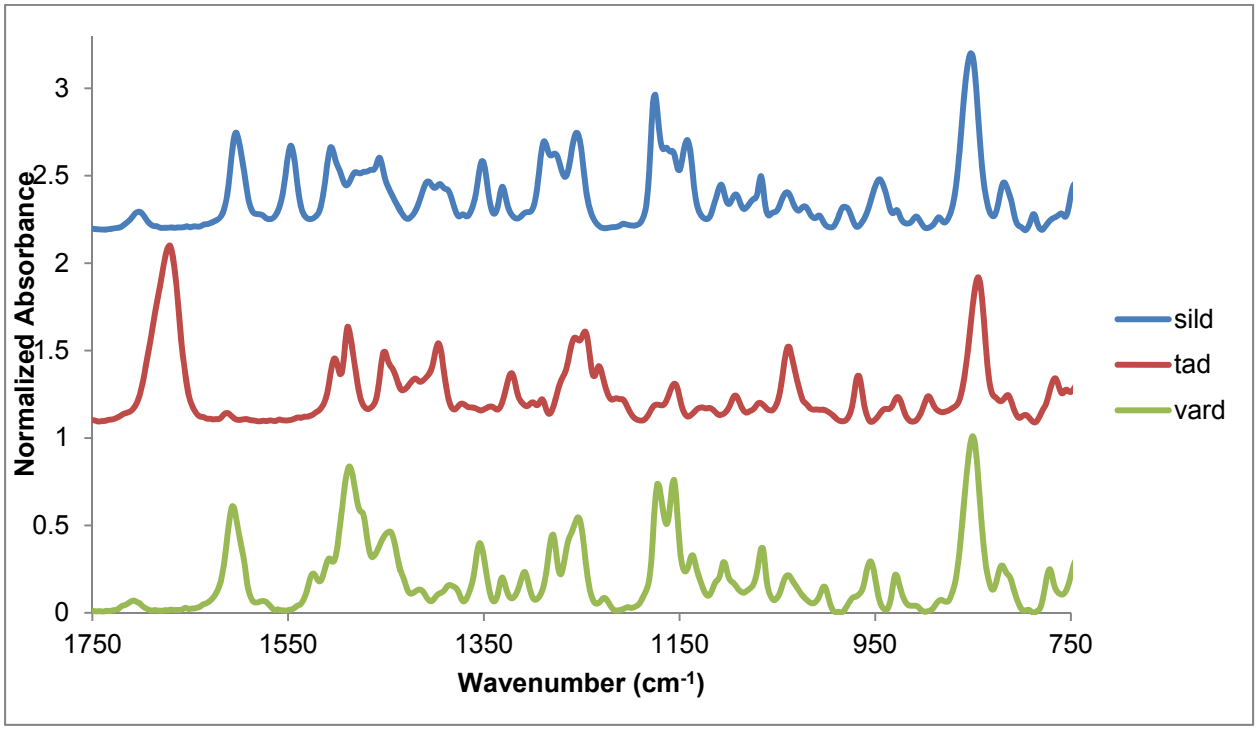

Figure 1.9. IR spectra for the derivatized analysis of tadalafil, sildenafil and vardenafil.

1.3.3. LOD and Linearity

Based on increased peak response, the limit of detection for the MS detector was determined using the derivatized peak response (Figure 1.4). Solutions of 7, 17, 34 and 85 µg/mL were analyzed as 1 µL aliquots. Because ¼ of the flow from the column is directed to the mass spectrometer, this correlates to 1.7, 4.3, 8.6 and 21 ng, respectively, going to the MS detector. At the 1.7 ng level, the signal to noise ratio was 3.29 for tadalafil, 5.29 for sildenafil and 1.43 for vardenafil. At the 4.3 ng level, the signal to noise ratio was 23.2 for tadalafil, 28.6 for sildenafil and 10.8 for vardenafil.

Based on response, the limit of detection and linearity for the IR detector were determined using the derivatized analysis as well. Because ¾ of the flow from the column is directed to the IR detector, this correlates to 5.1, 12.8, 25.7 and 64.1 ng, respectively, deposited onto the ZnSe disc. These deposits yielded the single wavenumber chromatograms shown in Figure 1.10 and the corresponding infrared spectra shown in Figure 1.11 for tadalafil (1671 cm^{-1}), sildenafil (1547 cm^{-1}) and vardenafil (1607 cm^{-1}), respectively. At the 5.1 ng level, the signal to noise ratio was 4.16 for tadalafil and 1.94 for sildenafil. Vardenafil was not detected at this concentration. At the 12.8 ng level, the signal to noise ratio was 16.7 for tadalafil, 5.19 for sildenafil and 5.72 for vardenafil. Using the extracted wavelength data, a calibration curve was made for each standard. The correlation coefficient for each analyte curve was 0.99 or better over a range of 5.1 to 64.1 ng for sildenafil and tadalafil, and a range of 12.8 to 64.1 ng for vardenafil.

Figure 1.10a: 1607cm^{-1} for TMS Sildenafil

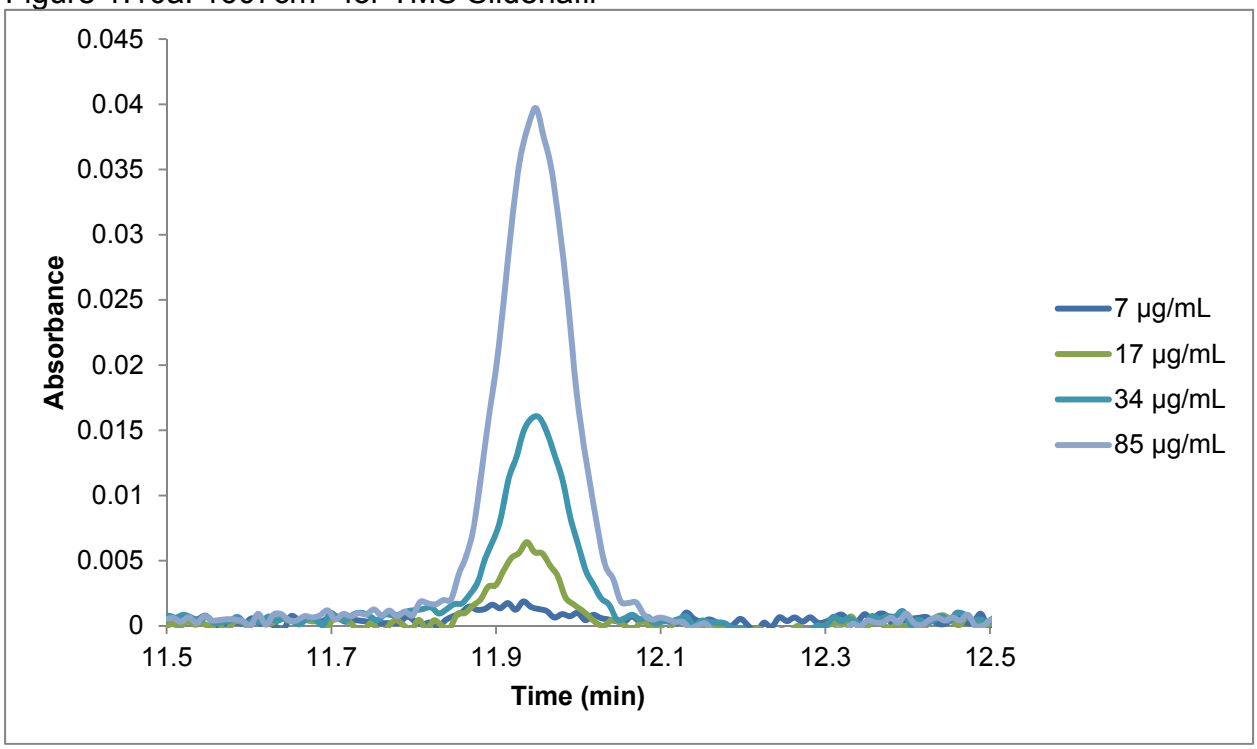

Figure 1.10b: 1607cm^{-1} for TMS Tadalafil

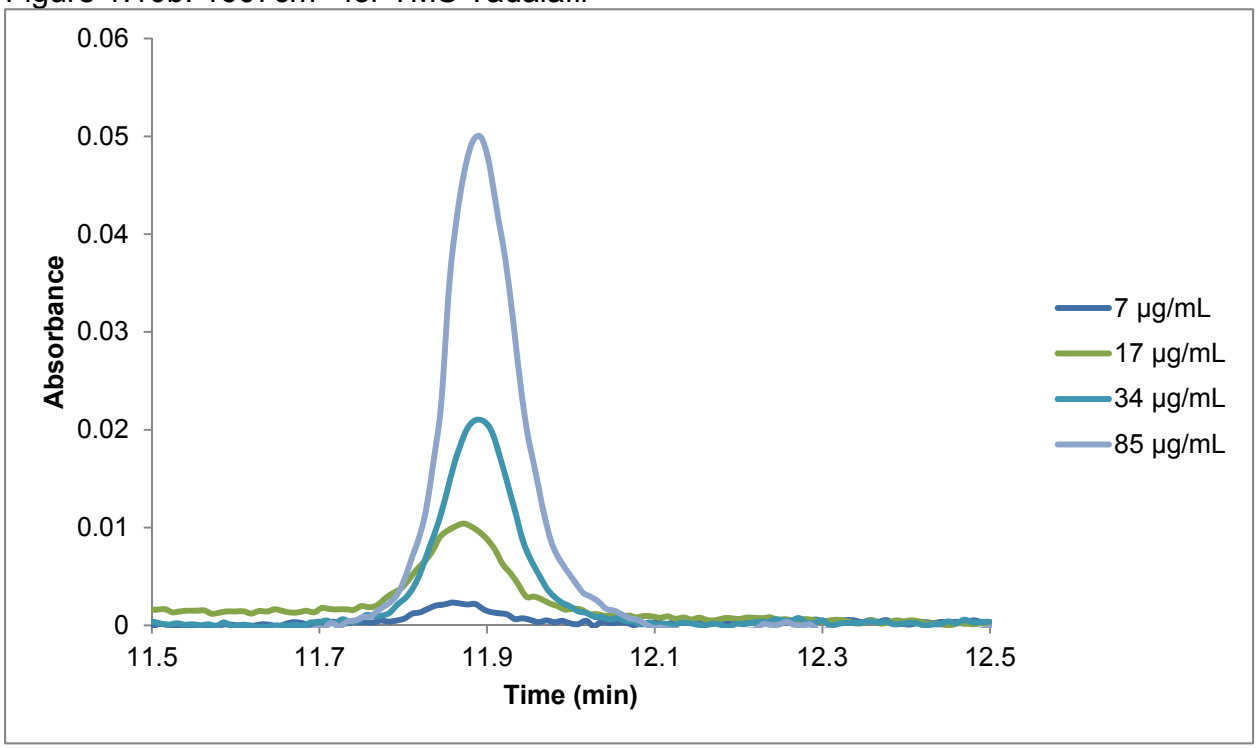

Figure 1.10c: 1607cm^{-1} for TMS Vardenafil

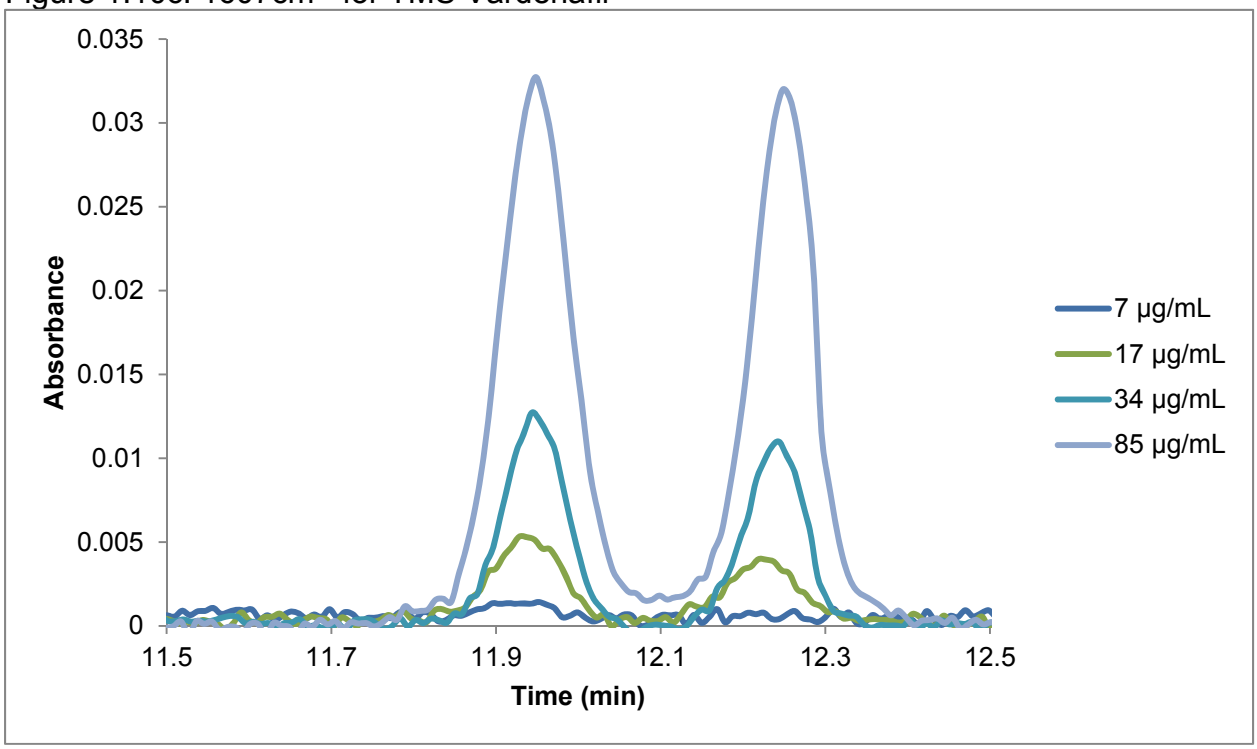

Figure 1.10. Extracted absorbance chromatograms for the derivatized analysis of tadalafil, sildenafil and vardenafil.

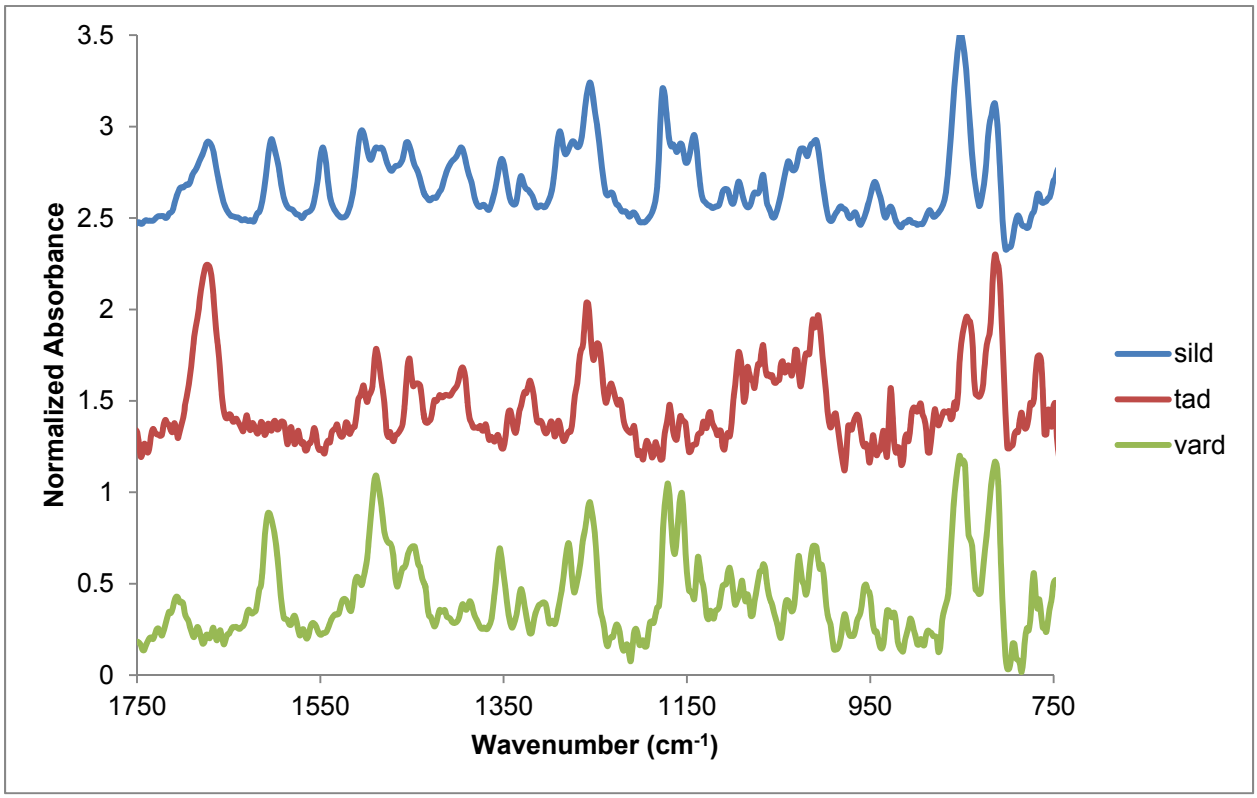

Figure 1.11. Infrared spectra of single wavenumber chromatograms

1.4. Conclusion

The recent merger of GC/MS with FT-IR provides advantages over each technique. This is both from a time and resources standpoint, but more importantly because the two techniques complement each other analytically. GC/MS is able to provide chromatographic separation and a molecular weight in addition to the presence of fragment ions. Ideally, a mass spectrum needs a minimum of three structurally significant ions for identification. Due to insufficient volatility, fragmentation, and loss of the molecular ion at lower concentrations for PDE-5 inhibitors, it is important to pair MS analysis with a scientifically uncorrelated technique for confirmation. FT-IR is capable of providing additional structural data related to functional groups, isomers and resonance.

It is worth noting that both techniques had similar LOD values and that these numbers are comparable to the LOD values of 1-2 ng for HPLC-UV and 0.1 – 1.4 ng for LCMS [1, 8]. Additionally, this method provides the ability to use both MS and IR spectral libraries for rudimentary identification and may prove highly beneficial in the analysis of other compounds that aren’t suitable for GC/MS due to their low volatility, which can be remedied by derivatization and optimization of the chromatographic parameters.

1.5. References

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Chapter 2 – Determination of Phosphodiesterase-5 (PDE-5) Inhibitors and Analogs using High-Performance Liquid Chromatography with Ultraviolet Detection

2.1. Introduction

The class of drugs known as phosphodiesterase-5 (PDE-5) inhibitors include sildenafil citrate (Viagra®, Pfizer), tadalafil (Cialis®, Eli Lilly) and vardenafil hydrochloride trihydrate (Levitra®, Bayer), and are marketed and approved for use in the United States in the treatment of erectile dysfunction (ED). During the last decade, these oral drugs have been the first-line treatment for ED as they are easily administered, relatively non-invasive, reversible, and well tolerated. Additionally, these drugs are generally preferred by patients than more invasive treatments, including injections and implants [1].

Approximately 50% of US men 40 – 70 years of age have reported experiencing ED [2]. Causes include certain medications, drug abuse, smoking, injury, obesity and the presence of comorbid conditions such as hypertension, heart disease, diabetes, depression or anxiety. Although PDE-5 inhibitors are beneficial for many who suffer from ED, the presence of certain comorbid conditions may make taking a PDE-5 inhibitor dangerous. Additionally, these drugs are known to have negative interactions with nitrates, alpha blockers and blood-thinning medication [3].

As a result, many men seek alternative medicines, in the form of herbal and dietary supplements, as a therapy for ED with the belief that these products are safe. Studies indicate that these products are often found to contain the active pharmaceutical ingredients (APIs) sildenafil, tadalafil or vardenafil [4-8] or analogs of these approved APIs [7, 9-29]. Furthermore, products containing multiple PDE-5

inhibitors have been found, both in the literature [7, 12, 20] and in samples analyzed by the U.S. Food and Drug Administration.

Currently, several methods are available to determine a single PDE-5 inhibitor or analog [4, 13, 18, 28, 30-33], and some are designed to assay more than one analyte [6-8, 26, 34-36] using either high-performance liquid chromatography with ultraviolet detection (HPLC-UV) or liquid chromatography-mass spectrometry (LC-MS). In cases where certain combinations of PDE-5 inhibitors and/or analogs are present in a single matrix, it may be necessary to run the sample by more than one method to determine the levels of analytes present. A single method able to analyze samples containing multiple PDE-5 inhibitors, and the large number of analogs detected to date, was desired.

In this report, a sensitive and accurate method capable of separating fourteen PDE-5 inhibitors and analogs in a variety of dosage forms is presented. Figures of merit for the various standards analyzed include linearity, dynamic range, precision, accuracy, limits of detection and quantitation, and ruggedness.

2.2. Experimental

2.2.1. Materials

Standard reference material for sildenafil citrate was provided by Pfizer Inc. (New York, NY), tadalafil by Eli Lilly and Company (Indianapolis, IN) and vardenafil hydrochloride trihydrate by Bayer Corporation (West Haven, CT). Standard reference materials for homosildenafil methanesulfonate, hydroxyhomosildenafil citrate, and sulfoaildenafil methanesulfonate were available as in-house standards, having been analytically verified by the Forensic Chemistry Center for use internally. Standards of

acetildenafil, aminotadalafil, dimethylsildenafil, hydroxyacetildenafil, hydroxythiohomosildenafil, pseudovardenafil, thiohomosildenafil, thiosildenafil and xanthoanthrafil were purchased from TLC PharmaChem Inc. (Ontario, Canada). HPLC grade CH_3CN, 0.1% trifluoroacetic acid (TFA) in H_2O and 0.1% TFA in CH_3CN were purchased from Fisher Scientific (St. Louis, MO). 18MΩ·cm deionized H_2O was generated using a Milli-Q system (Millipore, Billerica, MA). Disposable non-sterile luer lok syringes and 30 mm nylon syringe filters with 0.45 µm pore size were purchased from Fisher Scientific.

2.2.2 Standard Preparation

Stock standards were prepared at concentrations of approximately 0.2 mg/mL as the free base. Serial dilutions of the stock standard, down to 20 µg/mL were prepared to create a three-point calibration curve. The standards were made and diluted in CH_3CN:H_2O (50:50, v/v). Multiple analytes may be combined into a single solution or prepared separately.

For determination of analogs for which only a limited amount of standard is available, a single solution of the analyte of interest was prepared for the purpose of retention time comparison. To estimate quantity, a calibration curve was prepared using a standard that most closely resembled the analog in structure and UV spectral characteristics, as shown in Figure 2.1 and Table 2.1.

Figure 2.1a: Vardenafil and related analog

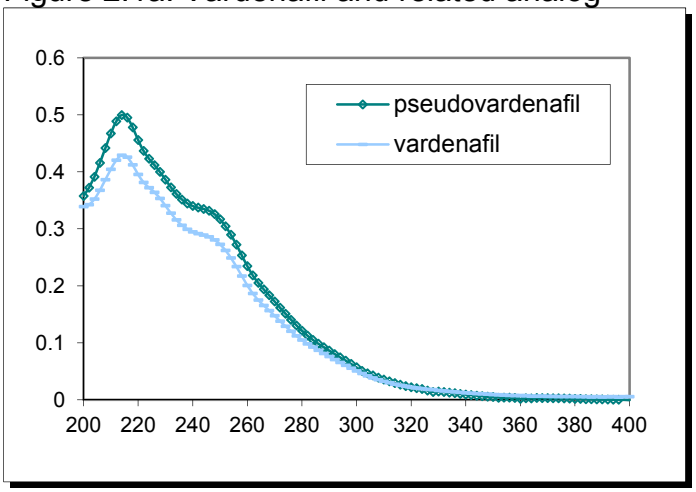

Figure 2.1b: Sildenafil and related analogs

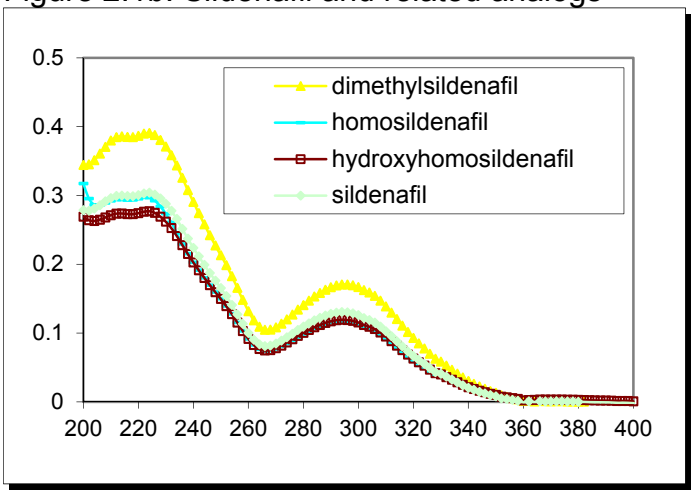

Figure 2.1c: Tadalafil and related analog

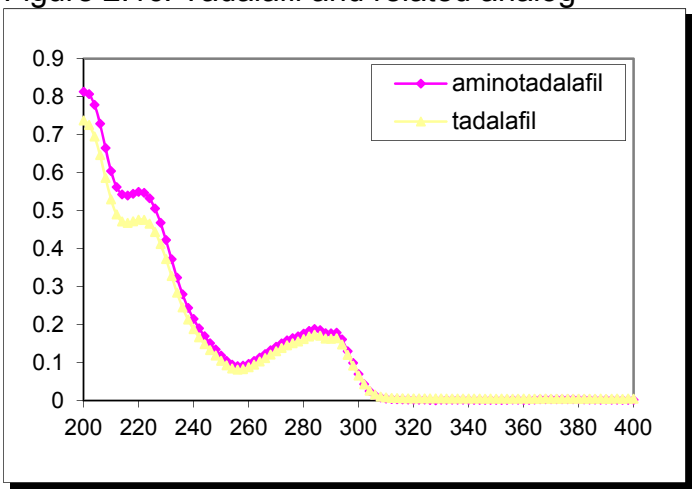

Figure 2.1d: Thione analogs

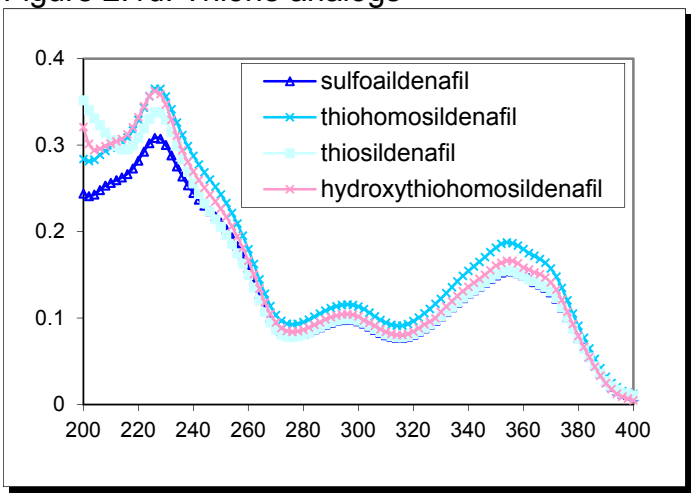

Figure 2.1e: Acetildenafil analogs

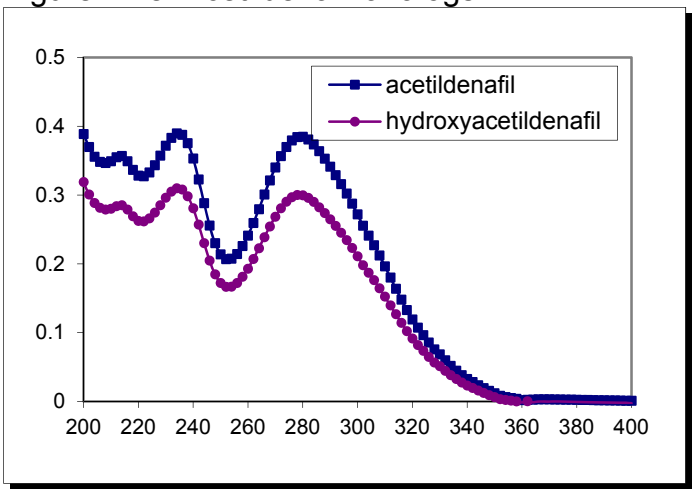

Figure 2.1f: Xanthoanthrafil

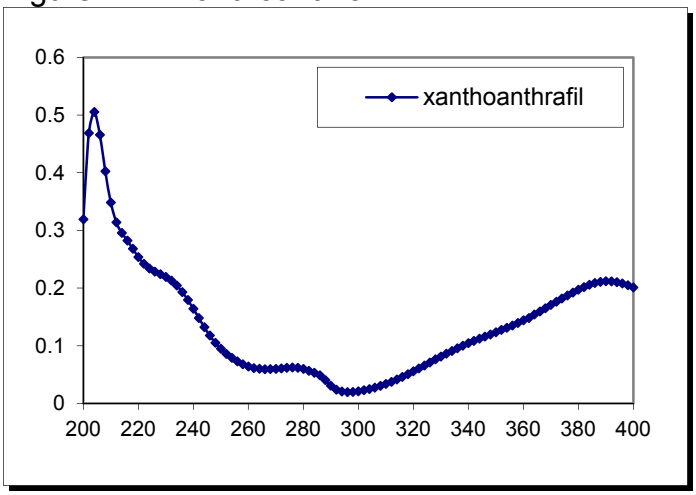

Figure 2.1. Absorbance spectra for 15 PDE-5 inhibitors and analogs collected using a Hewlett-Packard HP8452A diode array spectrophotometer. Samples were prepared in solutions of 50:50 CH_3CN:DI H_2O, and concentrations ranged from 4 to 7 µg/mL as the free base.

Table 2.1. Molar Absorptivities for PDE-5 Inhibitors and Analogs*

Name	Molar Absorptivity, $M^{-1} \cdot cm^{-1}$	
	285 nm	230 nm
Vardenafil	8611	32752
Pseudovardenafil	8382	31806
Hydroxyhomosildenafil	13196	31402
Sildenafil	12604	30161
Homosildenafil	11751	29689
Dimethylsildenafil	11520	27475
Hydroxythiohomosildenafil	9783	36211
Thiosildenafil	7724	28812
Thiohomosildenafil	9859	33934
Sulfoaildenafil	10291	35043
Aminotadalafil	12713	28708
Tadalafil	13403	29049
Hydroxyacetildenafil	23708	24537
Acetildenafil	24393	24588
Xanthoanthrafil	3962	17077

*Determined experimentally, see Figure 2.1.

2.2.3 Sample Preparation

A representative number of dosage units, generally 5 to 10 capsules or tablets, were combined to create a composite. When working with a sample that did not have a declared level, a portion equivalent to one-tenth to one-half tablet or capsule content weight was transferred to a suitably sized volumetric flask. Samples having a declared level of API were prepared such that the resulting analyte concentration was approximately 0.1 mg/mL. For both types of samples, the flasks were filled

approximately ½ full with CH_3CN:H_2O (50:50, v/v), shaken for 15 minutes using a mechanical shaker, diluted to volume and mixed. A portion of the extract was filtered.

For fortified samples, an additional portion of sample was prepared in the same manner as the initial sample preparation. Prior to adding solvent, the sample was fortified with an amount of appropriate standard equivalent to 50 – 150% of the amount determined to be present in the sample and thoroughly mixed to combine. If a sufficient quantity of an analog standard was not available, a standard was chosen that most closely resembled the analog. The fortified sample was then prepared as described above. If necessary, the filtrate was diluted further in order for the peak area response to fall within the calibration curve.

2.2.4 Selection of Detection Wavelength

For the selection of a suitable detection wavelength, standard solutions of 15 PDE-5 inhibitors and analogs were analyzed to determine absorbance. The absorbance spectra were collected using a Hewlett-Packard HP8452A diode array spectrophotometer (Agilent, Santa Clara, CA). Samples were prepared in solutions of CH_3CN:H_2O (50:50, v/v), and concentrations ranged from 4 to 7 µg/mL (Figure 2.1). The data show that most have absorbance maxima between 215 and 230 nm, and 280 and 300 nm. Vardenafil and pseudovardenafil do not have maxima in the region of 280 to 300 nm, but they do exhibit absorbance. The region of 285 nm, as opposed to a wavelength in the 215 to 230 nm range, is selective towards the analytes of interest, and exhibits reduced background absorbance. When necessary, detection at 230 nm allows for increased signal when analyzing low levels of analyte in various samples.

2.2.5 HPLC instrument parameters

Analyses were performed using a Waters 2690 Alliance System with a 996 PDA Detector, Empower Software v.2 from Waters Corporation (Milford, MA), and a Zorbax Eclipse XDB-C8, 150 mm x 4.6 mm, 5 µm column from Agilent Technologies (Santa Clara, CA). The mobile phase consisted of a 10 minute gradient method using Solvent A, 0.1% TFA in H_2O, and Solvent B, 0.1% TFA in CH_3CN. The gradient was performed as follows: 0 min, 25% B; 0–7 min, linear to 50% B; 3 min hold at 50% B. The column was equilibrated at 25% B for five minutes between runs. The flow rate was 1 mL/min, the injection volume was 10 µl, and the column temperature was ambient. The detection wavelength was 285 nm, with optional dual detection at 230 nm or online spectral collection from 210 to 400 nm as necessary. If a reference wavelength is used, care in its selection should be taken, as xanthoanthrafil and the thione analogs demonstrate strong absorbance at higher wavelengths (Figure 2.1).

2.2.6 Method Validation

The linearity and dynamic range of the method were evaluated using a series of standard solutions. Multiple injections of a wide range of concentrations of vardenafil, sildenafil, tadalafil, and the 12 analogs were used to construct calibration curves, and the correlation coefficient, y-intercept and slope were calculated. The average retention time and relative retention time to sildenafil were also determined.

Injection precision, limit of detection and limit of quantitation for each analyte were determined. One of the system suitability parameters that must be met in a typical USP monograph concerns the RSD for replicate injections. To mimic that requirement, a mid-level standard was injected five times. In order to determine limit of detection

(LOD) and limit of quantitation (LOQ), a low-level standard was injected seven times, and the standard deviation (SD) was determined. The LOD was defined as three times the SD, and the LOQ as ten times the SD, for each analyte.

Method accuracy and precision were assessed using dosage forms of Levitra®, Viagra® and Cialis®. Four preparations of each dosage form were evaluated using the gradient method, and the assay values were compared to those obtained using the manufacturer's methods for vardenafil, sildenafil and tadalafil. Additional method accuracy data from various dosage forms were determined by comparing assay values to those obtained using the manufacturer's methods.

Ruggedness of the method was determined using Waters, Dionex and Agilent HPLC systems, various C8 columns, and multiple analysts. Composites of Levitra®, Viagra® and Cialis® were analyzed, as were three dietary supplement capsule composites that were fortified with homosildenafil, hydroxyhomosildenafil or sulfoaildenafil.

2.3. Results

2.3.1 Specificity

The relative retention times for 15 PDE-5 inhibitors and analogs were determined. Initially, 14 of the analytes were combined into a single mixed standard and analyzed. Table 2.2 lists the average retention time and relative retention time, with respect to sildenafil, for the compounds evaluated. This includes the more recently obtained xanthoanthrafil, which was analyzed individually for inclusion with the mixed standard results.

Table 2.2. Retention Time and Relative Retention Time for the PDE-5 Inhibitors and Analogs

Name	Average Retention Time (minutes)	Relative Retention Time (relative to sildenafil)
Vardenafil	3.6	0.63
Hydroxyacetildenafil	3.8	0.65
Acetildenafil	4.0	0.70
Hydroxyhomosildenafil	5.6	0.96
Sildenafil	**5.8**	**1.00**
Homosildenafil	6.1	1.05
Dimethylsildenafil	6.2	1.08
Aminotadalafil	6.9	1.20
Tadalafil	8.1	1.41
Pseudovardenafil	8.1	1.41
Xanthoanthrafil*	8.3	1.42
Hydroxythiohomosildenafil	8.6	1.49
Thiosildenafil	9.0	1.55
Thiohomosildenafil	9.4	1.62
Sulfoaildenafil	9.5	1.64

*A standard for xanthoanthrafil was obtained recently. It was analyzed for retention time information, but it was not included in the mixture evaluated in Figures 2.2 and 2.3.

Table 2.3 summarizes the reproducibility of analyte retention times obtained over time using a single HPLC column. Multiple concentrations of standards of vardenafil, sildenafil, tadalafil, and the 12 analogs were analyzed in duplicate, at minimum, and both the within run and overall retention time data is shown. The RSD values for intra-day injections are typically less than 1.0%. The RSD values for inter-day analyses are typically less than 2.0%, indicating excellent system stability.

Table 2.3. Intra- and Inter-day Retention Time Reproducibility

Analyte		Number of Injections	Average Retention Time (min.)	Standard Deviation	Percent RSD
Vardenafil	Day 1	9	3.61	0.02	0.5
	Day 2	7	3.79	0.03	0.9
	Day 3	8	3.64	0.03	0.9
	Inter-day		**3.68**	**0.10**	**2.6**
Hydroxyacetildenafil	Day 1	6	3.93	0.02	0.4
	Day 2	7	4.01	0.05	1.2
	Day 3	8	3.86	0.06	1.6
	Inter-day		**3.93**	**0.08**	**1.9**
Acetildenafil	Day 1	6	4.16	0.02	0.6
	Day 2	7	4.26	0.03	0.8
	Day 3	8	4.07	0.04	0.9
	Inter-day		**4.16**	**0.10**	**2.3**
Hydroxyhomosildenafil	Day 1	9	5.55	0.02	0.3
	Day 2	7	5.79	0.02	0.4
	Day 3	8	5.59	0.02	0.4
	Inter-day		**5.64**	**0.13**	**2.3**
Sildenafil	Day 1	15	5.74	0.02	0.4
	Day 2	9	5.75	0.02	0.3
	Day 3	8	5.78	0.04	0.8
	Inter-day		**5.76**	**0.02**	**0.4**
Homosildenafil	Day 1	6	6.05	0.03	0.5
	Day 2	7	6.30	0.03	0.5
	Day 3	8	6.06	0.05	0.8
	Inter-day		**6.14**	**0.14**	**2.3**
Dimethylsildenafil	Day 1	6	6.42	0.05	0.8
	Day 2	7	6.50	0.08	1.2
	Day 3	8	6.34	0.06	1.0
	Inter-day		**6.42**	**0.08**	**1.2**
Aminotadalafil	Day 1	6	7.12	0.07	1.0
	Day 2	7	7.22	0.03	0.4
	Day 3	8	7.02	0.05	0.7
	Inter-day		**7.12**	**0.10**	**1.4**

Table 2.3. Intra- and Inter-day Retention Time Reproducibility (continued)

Analyte	Date	Number of Injections	Average Retention Time (min.)	Standard Deviation	Percent RSD
Tadalafil	Day 1	15	8.12	0.01	0.1
	Day 2	7	8.35	0.04	0.4
	Day 3	8	8.16	0.03	0.3
	Inter-day		**8.21**	**0.12**	**1.5**
Pseudovardenafil	Day 1	6	8.24	0.02	0.3
	Day 2	7	8.31	0.03	0.3
	Day 3	8	8.17	0.06	0.8
	Inter-day		**8.24**	**0.07**	**0.8**
Xanthoanthrafil	Day 1	6	8.48	0.02	0.3
	Day 2	7	8.41	0.03	0.4
	Day 3	8	8.23	0.04	0.4
	Inter-day		**8.37**	**0.13**	**1.5**
Hydroxythiohomosildenafil	Day 1	6	8.72	0.01	0.1
	Day 2	7	8.75	0.04	0.4
	Day 3	8	8.67	0.05	0.6
	Inter-day		**8.71**	**0.04**	**0.4**
Thiosildenafil	Day 1	6	9.10	0.03	0.4
	Day 2	7	9.14	0.02	0.3
	Day 3	8	9.07	0.05	0.6
	Inter-day		**9.11**	**0.03**	**0.4**
Thiohomosildenafil	Day 1	6	9.49	0.02	0.2
	Day 2	7	9.57	0.02	0.2
	Day 3	8	9.44	0.09	0.9
	Inter-day		**9.50**	**0.06**	**0.7**
Sulfoaildenafil	Day 1	9	9.40	0.06	0.6
	Day 2	7	9.73	0.05	0.6
	Day 3	8	9.51	0.06	0.7
	Inter-day		**9.55**	**0.17**	**1.8**

2.3.2 Linearity, Dynamic Range, LOD and LOQ

Calibration curves for the 15 analytes were constructed to determine linearity. Concentrations ranged from 1 to 10 µg/mL for the low standard, to 200 to 400 µg/mL for the high standard, and each curve covered at least two orders of magnitude. At least three concentrations of standards were used to create the calibration curve, and each standard was injected a minimum of three times. The correlation coefficient for each curve was calculated to be 0.999 or better. In Table 2.4, the RSD for injection precision for five injections of a 0.1 mg/mL solution was 0.4% or less for all of the analytes. To determine LOD and LOQ, a 1 µg/mL solution of each of the analytes was injected seven times. The calculated LOD was 0.2 µg/mL or less and the LOQ was 0.5 µg/mL or less.

2.3.3 Accuracy and Precision

For approved dosage forms, method accuracy and precision were determined by comparing assay values from the gradient method to the declared milligram per tablet level. Composites of the approved products were prepared, and four preparations of each composite were analyzed. For Levitra® 20 mg tablets, the average assay value was 20.4 $\pm$ 0.5 mg (102% declared). For Cialis® 10 mg tablets, the average value was 9.8 $\pm$ 0.2 mg (98% declared) and for Viagra® 100 mg tablets, the average value was 100 $\pm$ 1 mg (100% declared).

Additionally, duplicate preparations of various dosage forms were evaluated by comparing assay values to those obtained using the manufacturer's methods, as shown in Table 2.5. For sildenafil concentrations ranging from 15 to 130 milligrams per dosage unit, the result from the gradient method was within 94% of the value obtained from the

monograph [37]. For tadalafil concentrations ranging from 4.5 to 20 milligrams per dosage unit, the result was within 90% of the manufacturer's method.

Table 2.4. Values of Injection Precision, Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Analyte	Injection Precision, % RSD[a]	LOD, µg/mL[b]	LOQ, µg/mL[b]
Sildenafil	0.4	0.05	0.2
Tadalafil	0.2	0.05	0.2
Vardenafil	0.2	0.2	0.5
Acetildenafil	0.1	0.02	0.07
Aminotadalafil	0.2	0.03	0.1
Dimethylsildenafil	0.2	0.04	0.1
Homosildenafil	0.3	0.05	0.2
Hydroxyacetildenafil	0.3	0.04	0.1
Hydroxyhomosildenafil	0.3	0.01	0.03
Hydroxythiohomosildenafil	0.1	0.08	0.3
Pseudovardenafil	0.2	0.06	0.2
Sulfoaildenafil	0.3	0.05	0.2
Thiohomosildenafil	0.3	0.08	0.3
Thiosildenafil	0.1	0.06	0.2
Xanthoanthrafil	0.2	0.08	0.3

[a] To assess a typical system suitability requirement for RSD of replicate injections, a 0.1 mg/mL solution of each standard was injected five times.

[b] In order to determine the LOD and LOQ, a 1 µg/mL solution of each standard was injected seven times. The standard deviation (SD) of the seven injections was calculated. The LOD was defined as 3 x SD, and the LOQ was defined as 10 x SD.

Table 2.5. Comparison of Assay Results to Established Methods

Analyte	Matrix	Analyte Content, mg/dosage: Gradient method	Established Method[a,b]	Percent Difference
Sildenafil	Tablet composite	52	50	3.2
	Tablet composite	92	93	-1.7
	Tablet composite	59	58.6	0.7
	Tablet composite	97	99	-2.2
	Tablet composite	74	73	1.1
	Tablet composite	75	79	-5.7
	Capsule composite	60	61	-0.7
	Capsule composite	81	77	5.5
	Tablet composite	87.0	86.7	0.3
	Tablet composite	53	52	1.1
	Tablet composite	96	95.5	0.6
	Tablet composite	92	90	2.8
	Tablet composite	96	96	-0.2
	Tablet composite	130	129	1.0
	Tablet composite	16.2	15.8	2.6
	Tablet composite	14.9	15.0	-0.7
Tadalafil	Tablet composite	20.2	18.5	9.3
	Tablet composite	73	70.7	3.3
	Tablet composite	17	17.2	-1.0
	Tablet composite	20.3	19.1	6.2
	Tablet composite	19.2	18	4.2
	Tablet composite	18.5	19.5	-5.1
	Tablet composite	18.8	19.7	-4.6
	Tablet composite	20.1	20.4	-1.3
	Tablet composite	7.8	8.2	-5.4
	Tablet composite	8.8	9.1	-3.6
	Tablet composite	4.5	4.8	-5.9
	Tablet composite	9.4	10.1	-6.8
	Tablet composite	7.1	7.2	-0.5
	Tablet composite	8.4	8.2	2.3
	Tablet composite	7.6	7.5	1.2
	Tablet composite	20	19.1	4.5

[a] Established method for sildenafil: “In-Process Revision: Sildenafil Citrate” monograph, Pharmaceutical Forum (USP)

[b] Established method for tadalafil: “Determination of LY450190 (IC351) and Related Substances in Tablet Formulations by Reverse-Phase HPLC”, Eli Lilly and Company Laboratory Procedure B07000, Rev. 3, September 14, 1999.

Table 2.6. Spike Recovery Experiments

Analyte	Matrix	Analyte Content (mg/dosage)	Fortification Level (mg/dosage)	Percent Recovery
Sildenafil	Capsule composite	151	113	99
	Capsule composite	158	84	92
	Tablet composite	87	68	104
	Tablet composite	102	72	94
	Tablet composite	99	66	102
	Drink mix	33	29	97
	Bulk powder	684 mg/g	651 mg/g	94
	Tablet composite	16	14	98
	Tablet composite	15	21	101
	Sublingual strips	3 mg/strip	4 mg/strip	98
	Capsule composite	60	38	87
	Capsule composite	114	88	100
	Capsule composite	78	74	90
	Capsule contents	107	111	93
	Capsule contents	110	104	95
	Bulk powder	369 mg/g	380 mg/g	104
	Bulk powder	367 mg/g	394 mg/g	98
Tadalafil	Tablet composite	35	23	103
	Tablet composite	19	12	102
	Tablet composite	19	13	87
	Tablet composite	20	20	102
	Tablet composite	8	13	99
	Suspension	37 mg/mL	52 mg/mL	99
	Capsule composite	23	24	107
	Capsule composite	23	21	106
	Suspension	28 mg/mL	29 mg/mL	99
	Suspension	29 mg/mL	29 mg/mL	99
Sulfoaildenafil	Capsule composite	10	9	104
	Capsule contents	76	80	98
	Capsule composite	57	59	95
	Capsule composite	69	71	101
	Capsule composite	74	73	104
	Capsule composite	54	53	101
	Capsule composite	83	82	101
	Capsule composite	77	67	101
	Capsule composite	80	73	95
	Capsule composite	78	70	99

Table 2.6 shows recovery data for samples fortified with various levels of sildenafil, tadalafil and sulfoaildenafil. Recoveries ranged from 87 to 104% for sildenafil, from 87 to 107% for tadalafil, and 95 to 104% for sulfoaildenafil.

2.3.4 Ruggedness

To evaluate method ruggedness, composites of the three approved products and three dietary supplement capsule composites, each containing an analog, were analyzed using separate HPLC systems and multiple analysts, as outlined in Table 2.7. For Viagra® 50 mg tablets, the range in assay values was 47 to 51 mg. For Cialis® 20 mg tablets, the range was 19 to 20 mg and for Levitra® 10 mg tablets, the range was 9.3 to 9.9 mg. For a dietary supplement prepared to contain 72 mg homosildenafil per capsule, the range was 71 to 75 mg. For a prepared 43 mg sulfoaildenafil per capsule composite, the range was 44 to 45 mg, and for a prepared 59 mg hydroxyhomosildenafil per capsule composite, the range was 58 to 65 mg. It was also demonstrated that the assay values at 230 nm were comparable to the values for 285 nm.

2.4. Discussion

Prior to the development of this method, the determination of sildenafil, tadalafil or vardenafil was performed using individual methods. Sildenafil was determined using the Pharmaceutical Forum monograph for “Sildenafil Tablets” [37]. Tadalafil and vardenafil were determined using proprietary methods provided by the manufacturers. Because these methods required the preparation of unique mobile phases and sample diluents, and utilized different chromatographic columns and system parameters, it was problematic to analyze samples containing more than one PDE-5 inhibitor, particularly in cases where the amount of sample was limited.

Table 2.7. Evaluation of Method Ruggedness using Multiple Analysts

Matrix Content as Declared	Analyst[a]	Quantity Found, mg[b]	
		230 nm	285 nm
Viagra® 50 mg sildenafil per tablet	Analyst 1	47	47
	Analyst 2	51.2	51.2
	Analyst 3	49.8	49.9
	Average[c]	49.3	49.3
	SD	2.2	2.2
	RSD	4.4	4.4
	95% CI	2.3	2.3
	% Declared	98.6	98.6
Cialis® 20 mg tadalafil per tablet	Analyst 1	20.2	20.1
	Analyst 2	19.4	19.3
	Analyst 3	19.6	19.5
	Average[c]	19.7	19.6
	SD	0.4	0.4
	RSD	2.0	2.0
	95% CI	0.4	0.4
	% Declared	98.5	98.0
Levitra® 10 mg vardenafil per tablet	Analyst 1	9.35	9.34
	Analyst 2	9.94	9.94
	Analyst 3	9.51	9.59
	Average[c]	9.6	9.6
	SD	0.3	0.3
	RSD	2.8	2.9
	95% CI	0.3	0.3
	% Declared	96.0	96.2

Table 2.7. Evaluation of Method Ruggedness using Multiple Analysts (continued)

Matrix Content as Prepared	Analyst[1]	Quantity Found, mg[2]	
		230 nm	285 nm
Dietary Supplement 72 mg homosildenafil per capsule	Analyst 1	75	75
	Analyst 3	74.3	74.5
	Analyst 4	71	71
	Average[3]	73.6	73.5
	SD	3.3	3.3
	RSD	4.5	4.4
	95% CI	3.5	3.4
	% Declared	102	102
Dietary Supplement 43 mg sulfoaildenafil per capsule	Analyst 1	44.4	44.4
	Analyst 2	44.6	44.4
	Analyst 3	45	45
	Analyst 4	44.1	44.4
	Average[3]	44.4	44.4
	SD	0.5	0.5
	RSD	1.2	1.1
	95% CI	0.4	0.4
	% Declared	103	103
Dietary Supplement 59 mg hydroxyhomosildenafil per capsule	Analyst 1	65.2	65.1
	Analyst 2	64.0	63.8
	Analyst 3	59	59
	Analyst 4	58.3	58.1
	Average[3]	61.7	61.6
	SD	3.2	3.3
	RSD	5.3	5.4
	95% CI	2.7	2.8
	% Declared	105	104

[1] Analyst 1: Waters 2690 Alliance, 996 PDA detector; Zorbax Eclipse XDB-C8, 4.6 x 150 mm
Analyst 2: Waters 2690 Alliance, 996 PDA detector; Zorbax Eclipse XDB-C8, 4.6 x 150 mm
Analyst 3: Dionex Summit, PDA 100 detector; Waters XTerra RP8, 4.6 x 150 mm
Analyst 4: Agilent 1200 Series HPLC, DAD detector; Zorbax SB-C8, 4.6 x 150 mm

[2] Duplicate preparations of each matrix were analyzed. The value reported is the average of the two preparations.

[3] The values for average, standard deviation (SD), relative standard deviation (RSD), 95% confidence interval (CI) and percent declared were calculated using all analysts' preparations.

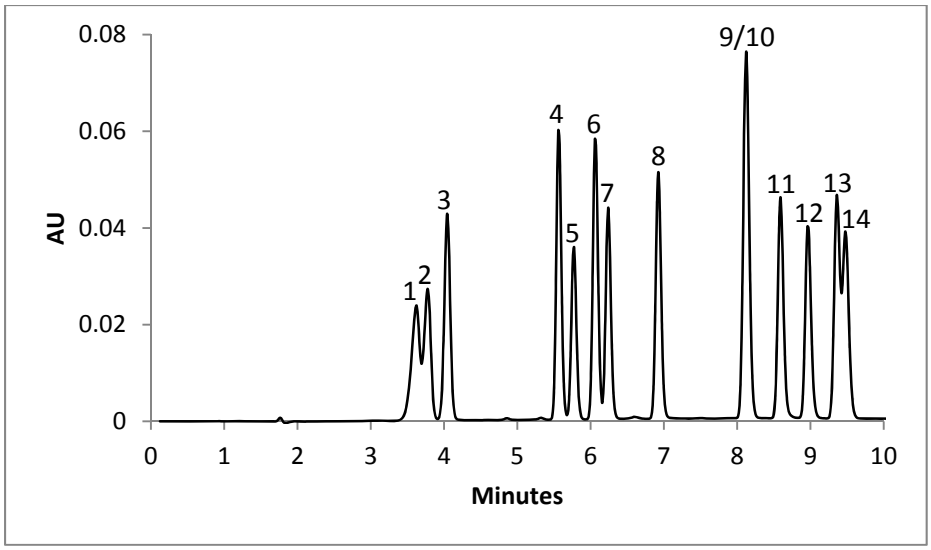

Figure 2.2. Chromatogram of 14 PDE-5 inhibitors and analogs. (1) vardenafil, (2) hydroxyacetildenafil, (3) acetildenafil, (4) hydroxyhomosildenafil, (5) sildenafil, (6) homosildenafil, (7) dimethylsildenafil, (8) aminotadalafil, (9) tadalafil, (10) pseudovardenafil, (11) hydroxythiohomosildenafil, (12) thiosildenafil, (13) thiohomosildenafil and (14) sulfoaildenafil. Note that tadalafil and pseudovardenafil co-elute. Concentration values for the free base ranged from 28 µg/mL for acetildenafil, to 135 µg/mL for thiosildenafil.

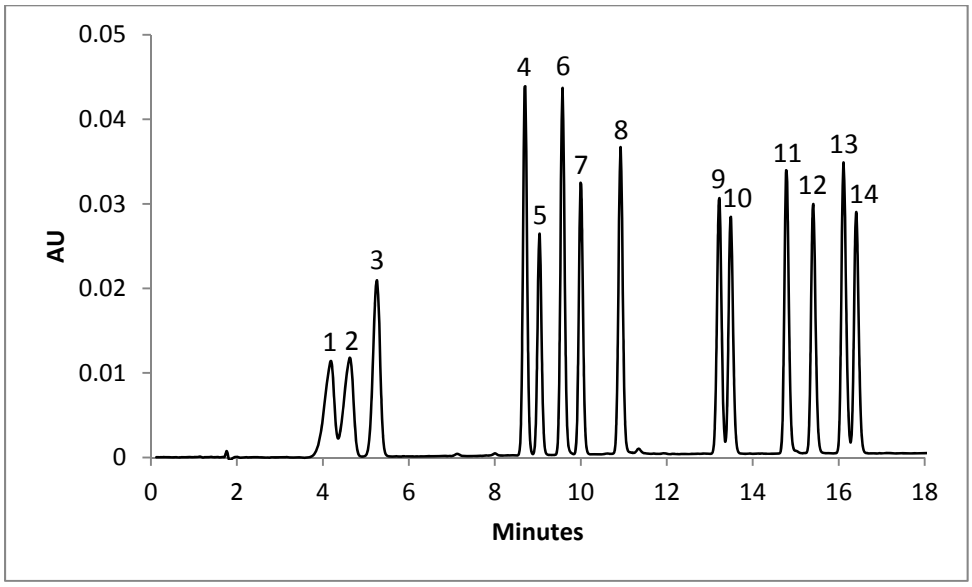

Figure 2.3. Chromatogram of 14 PDE-5 inhibitors and analogs, using the extended gradient method. The extended gradient program consisted of an 18 minute run using Solvent A, 0.1% TFA in H_2O, and Solvent B, 0.1% TFA in CH_3CN. The gradient was performed as follows: 0–2 min, 25% B; 2–17 min, linear to 50% B, 1 min hold at 50% B. The column was equilibrated at 25% B for five minutes between runs. All other experimental parameters are the same. See Figure 2.2 for numerical designations.

Before the samples were assayed, they were analyzed using LC-MS for identification purposes [5]. Using the chromatography parameters of the LC-MS method as a guide, it was determined that a similar method could be used for the quantification of PDE-5 inhibitors and analogs. The resulting gradient program can separate 14 of the 15 PDE-5 inhibitors and analogs investigated within 10 minutes, as shown in Figure 2.2. Tadalafil and pseudovardenafil cannot be separated chromatographically using these parameters, but they can be distinguished by their UV spectra. If complete separation is necessary, the gradient can be extended to 18 minutes, as shown in Figure 2.3. This extended gradient method also provides greater resolution for the pairs of vardenafil and hydroxyacetildenafil, homosildenafil and dimethylsildenafil, and thiohomosildenafil and sulfoaildenafil. Figure 2.4 is an example of using the faster gradient method to analyze two PDE-5 inhibitors, sildenafil and tadalafil, present in a single sample.

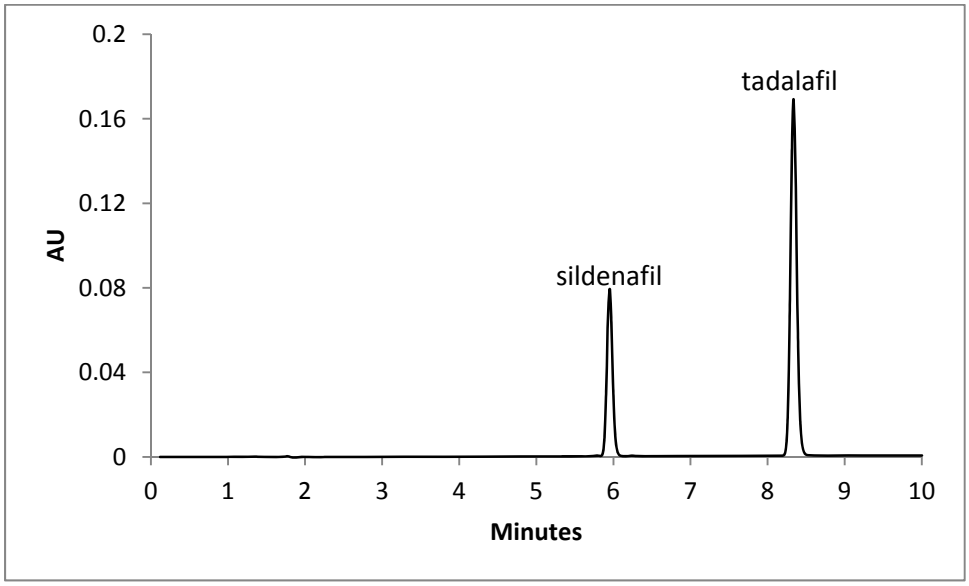

Figure 2.4. Chromatogram of sildenafil and tadalafil using the gradient method in Figure 2. Both PDE-5 inhibitors were present in a single tablet formulation. A single set of prepared extractions was used to assay both compounds.

The PDE-5 inhibitors and analogs analyzed were grouped into six distinct classes based on their UV spectra, as shown in Figure 1. Using these classes, suitable standards for estimation, when the specific analyte is not available or is cost-prohibitive for infrequent analysis, can be selected more easily. Additionally, if a standard for a particular analog was not available, peak identification may be determined by UV spectrum and LC-MS elution order comparisons.

2.5. Conclusion

This method provides separation of 14 PDE-5 inhibitors and analogs in a variety of forms, including tablets, capsules, bulk powders, troches, and liquids, in ten minutes. It is linear over a wide range of concentrations, sensitive to trace levels, accurate in dosage forms as well as supplements, and rugged across analysts and instruments. Additionally, by expanding the gradient method to an 18 minute run time, all 15 compounds can be resolved, offering the flexibility needed to separate and analyze new analogs as they are encountered.

Additionally, UV data presented indicate that PDE-5 inhibitors with similar structures have similar spectra and similar molar absorptivities. This proved useful in the latest characterization of two new tadalafil analogs, 2-hydroxypropylnortadalafil and n-butylnortadalafil [38], as they have spectra similar to tadalafil. As the work in this field of study continues, it may be difficult to maintain an up-to-date collection of each standard needed to perform quantitative analysis and the ability to use a spectrally-related compound to provide estimated levels is helpful in cases where the procurement of a standard is difficult, time consuming or cost prohibitive.

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Chapter 3 – Isolation and Characterization of a Methamphetamine Analog in a Dietary Supplement and Determination of Substituted Phenethylamines using High-Performance Liquid Chromatography with Ultraviolet Detection

3.1 Introduction

Substituted phenethylamines are central nervous system stimulants that speed up the various systems of the body. They work as a neuromodulator or neurotransmitter by releasing norepinephrine and dopamine in the brain. A drug from this class was first marketed as an over-the counter inhaler in the 1930's to treat nasal congestion, but over the years these drugs have been found to also help those suffering from sleeping disorders, narcolepsy or attention-deficit hyperactivity disorder, and can be used as an appetite depressant, vasoconstrictor, bronchodilator or calcium channel blocker. However, because many of these drugs come with a high potential for abuse and limited medical use, most of the drugs in this category are considered Schedule II stimulants by the DEA, and are subject to stringent procedures for refill by prescription [1-3].

Often abused as a recreational drug because of the euphoric rush many users experience, the effects are similar to those of cocaine but generally last longer because it is metabolized more slowly. Overdose and abuse can lead to agitation, an increase in body temperature, auditory and visual hallucinations, convulsions, violent and erratic behavior, paranoia, dental problems, issues with pregnancy and the fetus, brain damage and a psychosis similar to schizophrenia when chronically abused. Suicidal thoughts have been reported, and death from stroke, heart attack or overheating can also occur [3-7].

Due to a relatively easy synthesis process and multiple available routes of synthesis, these drugs can be made in small clandestine laboratories, using relatively inexpensive ingredients. These include batteries, matches or road flares, and pseudoephedrine, a common ingredient in cold medications. In more recent years, the legal sale of pseudoephedrine has been recorded, to limit the amount that any one person can purchase at a time [8, 9].

Additionally, these labs are always working to stay one step ahead of detection. This includes continuous development of new analogs, which are often modifications of any part of the base structure (the phenyl ring, side chain or amino group), and can therefore include any number of mixtures or structurally related contaminants. The resulting products can then be easily purchased on the internet, and it is difficult to know the effects these designer drugs may have on consumers without any FDA or DEA regulation [6, 10-14].

Given the variety of substances on the market, and that they are closely related in chemical structure, identification is of the utmost importance. Various techniques have been employed, including gas chromatography with mass spectrometry (GC/MS) [15, 16], high performance liquid chromatography with ultraviolet detection (HPLC-UV) [17], liquid chromatography with mass spectral detection (LC/MS) [18, 19], capillary zone electrophoresis (CE) [20, 21], and Fourier-transform infrared spectroscopy (FTIR) [22]. However, single technique identification can prove difficult, as in the case of N,N-dimethylamphetamine and N-ethylamphetamine, which have the same molecular weight. The mass spectra and ultraviolet spectra are relatively indistinguishable, but can be easily differentiated using chromatography or the infrared and nuclear magnetic

resonance (NMR) spectra. Other methods use a combination of multiple techniques, including those mentioned above, along with techniques such as Raman spectroscopy, thin layer chromatography, Time of Flight mass spectrometry (ToF), and gas chromatography with direct deposit Fourier transform infrared detection and mass spectrometric detection (GC/IR/MS) [23-25]. Recently, several dietary supplement samples were analyzed for substituted phenethylamines and a new analog was discovered. It was characterized using GC/MS, LC ToF and NMR, and determined to be N-ethyl-α-ethylphenethylamine when compared to a bulk powder of the same material, seized in transit from Vietnam to South Korea [5].

This research focuses on a specific analog, detected in several dietary supplements and identified as N-ethyl-α-ethylphenethylamine. It is structurally similar to methamphetamine [26], and assay using HPLC-UV indicated its presence at a significant level in the collected samples. Additionally, the dietary supplements also contained phenethylamine, and so a method was developed to quantitatively determine structurally related compounds in this category simultaneously. As the search for the next blockbuster “designer drug” continues by corrupt manufacturers, this type of isolation, characterization and determination work is vitally important toward the goal of protecting public health.

3.2 Experimental

3.2.1. Materials

Standard reference material for 2-phenethylamine hydrochloride was purchased from Acros Organics (Fair Lawn, NJ), N,N-dimethylphenethylamine was purchased from Sigma-Aldrich (St. Louis, MO), (±)-methamphetamine in methanol (1mg/mL) was

purchased from Cerilliant (Round Rock, TX), N-ethyl-α-ethylphenethylamine was purchased from ELSohly Laboratories, Inc (Oxford, MS) and phentermine hydrochloride was purchased from the US Pharmacopeial Convention, Inc. (Rockville, MD).

Chloroform, HPLC grade methanol, HPLC grade acetonitrile, hydrochloric acid, phosphoric acid, and sodium hydroxide were purchased from Fisher Scientific (St. Louis, MO). 18MΩ·cm deionized H_2O was generated using a Milli-Q system (Millipore, Billerica, MA).

NMR Tubes 5mm Wilmad LabGlass 7" Economy Tubes (500 MHz rated), disposable non-sterile luer lok syringes, 30 mm nylon syringe filters with 0.45 µm pore size and 15 mL conical tubes were purchased from Fisher Scientific.

3.2.2 Extraction Procedure

Approximately 10 g of each drink mix was transferred to stoppered Erlenmeyer flasks. 50 mL of 0.1N HCl was added to each flask and sonicated for 30 minutes. The solutions were divided into 15 mL conical tubes and centrifuged for 15 minutes at 5000 rpm, transferred to beakers and 5 mL of 12N NaOH was added with gentle mixing. Then, 25 mL of chloroform was added with mixing and solutions were transferred to separatory funnels. Funnels were allowed to sit overnight before the chloroform layer was collected. 10mL of 1.2N HCl was added to the chloroform extracts and sonicated for 15 minutes. Solutions were transferred to separatory funnels and the chloroform layer was discarded. The acidic layer was collected and dried down under a stream of nitrogen to reduce the volume in half. The resulting solution was analyzed by fraction collection, consolidated and dried down at 50°C and 20% speed in a vacuum evaporator, resulting in approximately 23 mg of N-ethyl-α-ethylphenethylamine

collected. The dried film was brought up in 1 mL CH_3CN and sonicated to dissolve. The resulting solution was analyzed using HPLC-UV to confirm the peak of interest and detect any possible contaminants. The remaining solution was then dried again for further characterization using Exact Mass Determination, LC-MS and NMR.

3.2.3 HPLC-UV

Fraction collection was performed using an Agilent 1200 Series HPLC with diode array detector and fraction collector, ChemStation for LC 3D Systems Software Rev. B.04.01 from Agilent Technologies (Santa Clara, CA), and a Waters Nova-Pak C18 150mm x 3.9 mm, 4 µm column from Waters Corporation (Milford, MA). The mobile phase consisted of a 5 minute isocratic method using 20% CH_3CN with a 1.5 mL/min flow rate, a 200 µl injection volume, ambient column temperature and detection wavelength of 258 nm (with spectral collection from 210 to 400 nm). The eluent was collected in an autosampler vial from 2.4 to 3.5 minutes, and diverted to waste for the remainder of the run (Figure 3.1).

Initial analyses were performed using a Waters 2690 Alliance System with a 996 PDA Detector, Empower Software v.2 from Waters Corporation, and a Phenomenex Prodigy ODS3, 250 mm x 4.6 mm, 5 µm column from Phenomenex (Torrance, CA). The mobile phase consisted of a nine minute gradient method using Solvent A, 75:25 9.2 g/L monobasic sodium phosphate in H_2O taken to pH 3.0 with phosphoric acid and methanol, and Solvent B, MeOH. The gradient was performed as follows: 0-4 min, 0% B; 4–7 min, linear to 30% B; 7-9 min at 0% B (equilibration time). The flow rate was 1.5 mL/min, the injection volume was 10 µl, and the column temperature was 40°C. The detection wavelength was 258 nm, with online spectral collection from 210 to 400 nm.

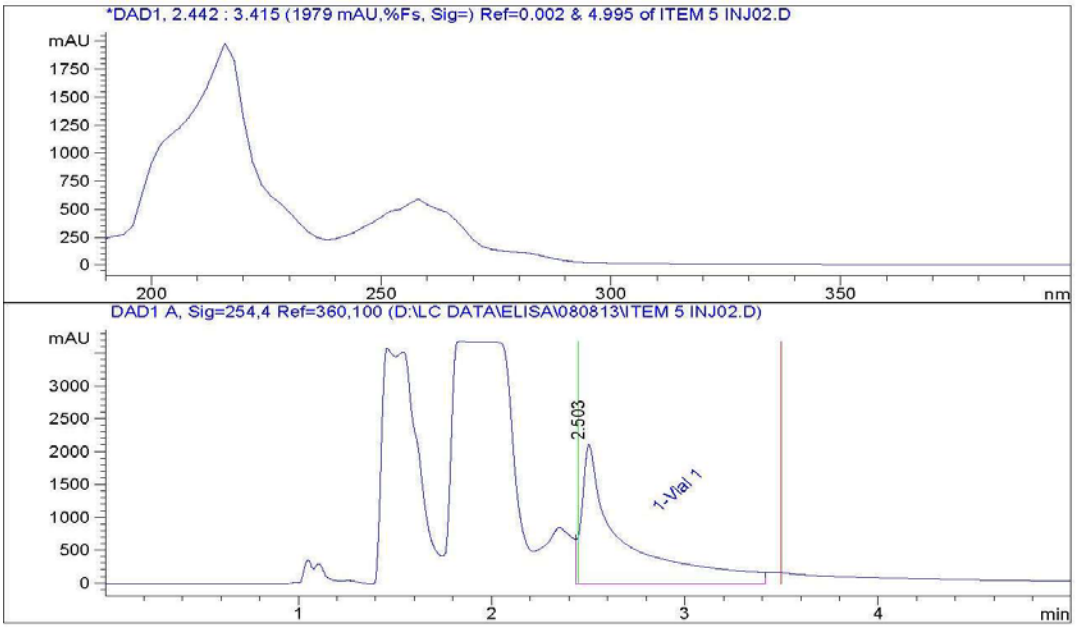

Figure 3.1. Example of HPLC fraction collection chromatogram and UV spectrum

Subsequent analysis of the standards within the developed gradient method was performed using a Waters 2690 Alliance System with a 996 PDA Detector, Empower Software v.2 from Waters Corporation, and a Zorbax Rx-C18, 250 mm x 4.6 mm, 5 µm column from Agilent Technologies. The mobile phase consisted of a 15 minute gradient method using Solvent A, 9.2 g/L monobasic sodium phosphate in H_2O taken to pH 3.0 with phosphoric acid, and Solvent B, MeOH. The gradient was performed as follows: 0 min 10% B, 0-6 min, linear to 15% B; 6-11 min, linear to 50% B and 11-12.5 min, linear to 90% B. The column was equilibrated at 10% B for five minutes between runs. The flow rate was 1.5 mL/min, the injection volume was 15 µl, and the column temperature was 40°C. The detection wavelength was 258 nm, with online spectral collection from 210 to 400 nm.

3.2.4 Orbitrap MS

Accurate mass analysis was performed using a Thermo Scientific Exactive Orbitrap equipped with an Ion Max Source. Instrument control and data collection are controlled by Xcalibur Software Version 2.1 from Thermo Scientific (Waltham, MA). The sample was directly infused into the instrument using a Chemyx Fusion 100 syringe pump and the syringe included in the instrument accessory kit. The mass spectrometer parameters were as follows: ionization = positive, electrospray; sheath gas flow = 10 arbitrary units; auxiliary gas flow = 0 arbitrary units; sweep gas flow = 0 arbitrary units; spray voltage = 3.5 kV; capillary temperature = 250°C; probe position = C,0,1.0; syringe flow rate = 10 µL/min; scan range *m/z* 120-250 (full scan); resolution 100,000 @ 1 Hz, AGC target = 1,000,000; max ion inject time = 500 ms

3.2.5 LC-MS

Analysis was performed using an Agilent 1200 Series Liquid Chromatograph, using a Zorbax SB C18 50 mm x 2.1 mm, 1.8 µm column from Aglient. Mobile phase A consisted of 0.1% formic acid in DI water and B was acetonitrile. From 0 to 2 minutes, the gradient was a linear change from 5% B to 95% B, and held for six minutes with a 3 minute post time. The flow rate was 350 µL/min, the injection volume was 1 µl, the sample compartment was at ambient temperature and the column temperature was 40°C. The HPLC was coupled to a Thermo Finnigan LTQ equipped with a Thermo Finnigan Ion Max source, analyzed in the ESI+ mode. The mass spectrometer parameters were as follows: ionization = positive, electrospray; sheath gas flow = 50 arbitrary units; auxiliary gas flow = 15 arbitrary units; sweep gas flow = 15 arbitrary units; spray voltage = 3.5 kV; capillary temperature = 275°C; acquisition time = 6.0

minutes; divert valve 0.0 min to waste, 0.1 min to source, 5.8 min to waste; start delay = 0.2 minutes; probe position = D,0,1.0 and with additional parameters and described in Table 3.1. Instrument control and data collection for both the HPLC and LTQ were controlled by Xcalibur software version 2.07.

Table 3.1. LC-MS scan event parameters

Parameter	Scan Event 1	Scan Event 2	Scan Event 3	Scan Event 4	Scan Event 5
Scan Range	*m/z* 85-650	N/A	N/A	N/A	N/A
Precursor Ion Mass	N/A	*m/z* 178.1	178.1→133	122.1	122.1→105.1
Precursor Ion Isolation Width	N/A	2.0	2.0	2.0	2.0
Collision Energy	N/A	25%	25%	30%	30%
Product Ion Scan Range	N/A	50-225	50-225	50-150	50-150
Wideband Activation	N/A	Off	Off	Off	Off

3.2.6 GC-MS

An Agilent Technologies GC 6890N Series with 7683 Series Autosampler and Restek (Bellefonte, PA) Rtx-5MS w/ Integra Guard 36.7 meter, 0.25mm ID and 0.25 micron film thickness column, coupled to an Agilent Technologies 5973N Mass Selective Detector was used to collect GC-MS data. The helium carrier gas in constant flow mode had a pressure of 9.79 psi, 0.9 mL/min flow rate and 31 cm/sec average velocity. Injection parameters: 1:20 split with a 1 µl injection volume and temperature of 250°C. GC oven gradient parameters: 75°C start temperature, 1.0 min hold, ramp at 12°C/min to a final temperature of 330°C, held for 12.75 minutes, for a 35.0 min run

time. The GC auxiliary temperature was 280°C. MS parameters: electron impact ionization; full scan mode; filament delay = 3.5 minutes (5.5 minutes for derivatized analyses); mass range = 40-550; threshold = 150; sampling = 2; MS source temperature = 230°C and MS quad temperature 150°C. Instrument control and data collection for both the GC and MS were controlled by Agilent ChemStation version E.02.02.1431. Spectra were compared to the Wiley Registry of Mass Spectral Data, 7th Edition and Designer Drug Library 2011 from John Wiley & Sons (Hoboken, NJ), NIST08 Mass Spectral Library from the National Institute of Standards and Technology (Gaithersburg, MD).

Sample extracts were derivatized by evaporating 40 µl of each extract to dryness in an autosampler vial, at 70°C under a stream of nitrogen. Then, 200 µl pyridine and 200 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added to the vial before capping tightly and incubating for 30 minutes at 70°C before analyzing. For ease, the entire reaction can be performed in the original autosampler vial and then directly analyzed.

3.2.7 NMR

NMR spectra were recorded at 25°C in $CDCl_3$ at 500 MHz (proton frequency) using a Bruker AVANCE III 500 High Resolution NMR spectrometer equipped with a 5 mm BBFO BB-1H/D Z-GRD from Bruker (Billerica, MA). Instrument was equipped with a Bruker BioSpin Ultrashield PLUS Superconducting Magnet, 5.4 cm bore and an operation field @ 11.7 Tesla. The probe temperature was 298K. One-dimensional experiments included 1H and 13C. Two-dimensional experiments included 1H—1H

correlated spectroscopy (1H—1H COSY) and edited heteronuclear single quantum coherence (HSQC). Data was recorded using Bruker TopSpin v. 2.1 PL 4 software.

3.2.8 Standard Preparation

Stock standards were prepared at concentrations of approximately 0.1 mg/mL as the free base. Serial dilutions of the stock standard, down to 50 µg/mL were prepared to create calibration curves. The standards were prepared in, and diluted with, methanol. Multiple analytes may be combined into a single solution or prepared separately.

3.2.9 Sample Preparation

Five gram portions, prepared in triplicate, were sampled directly from the product containers into scintillation vials. Based on estimated levels from GC-MS analysis, 10 mL of methanol was added to each vial, vortexed to mix and sonicated in a water bath for 30 minutes. A portion of the extract was filtered prior to analysis.

For fortified samples, an additional portion of sample was prepared in the same manner as the initial sample preparation. Prior to adding methanol, the sample was fortified with an amount of appropriate standard equivalent to 50 – 150% of the amount determined to be present in the sample and thoroughly mixed to combine. The fortified sample was then prepared as described above. If necessary, the filtrate was diluted further in order for the peak area response to fall within the calibration curve.

2.2.10 Method Validation

The linearity and dynamic range of the method were evaluated using a series of standard solutions. Multiple injections of a wide range of concentrations of 2-phenethylamine, N,N-dimethylphenethylamine, methamphetamine, N-ethyl-α-

ethylphenethylamine, and phentermine were used to construct calibration curves, and the correlation coefficient, y-intercept and slope were calculated. The average retention time and relative retention time to 2-phenethylamine were also determined.

3.3 Results and Discussion

3.3.1 HPLC-UV Analysis

The isolation of N-ethyl-α-ethylphenethylamine utilized retention time as the parameter for fraction collection. Product was isolated from two related samples, and the recovery from approximately ten grams of Samples A and B was 7 mg and 16 mg of N-ethyl-α-ethylphenethylamine, respectively. Comparison of the UV spectra from Samples A and B characterized the isolated fraction as consistent with 2-phenethylamine (Figure 3.2), in addition to the spectra provided in the literature for the N-ethyl-α-ethylphenethylamine analog [5].

Several powdered drink mix samples, representing three product flavors, were analyzed for N-ethyl-α-ethylphenethylamine and 2-phenethylamine content by comparing the peak areas to those obtained from a series of standard solutions. The amount of N-ethyl-α-ethylphenethylamine present was determined to be approximately 4 mg/g, which is equivalent to 21-25 mg per serving, based on the suggested serving size. The amount of 2-phenethylamine present was determined to be approximately 12 mg/g, which is equivalent to 62-70 mg per serving, based on the suggested serving size. The spike recovery for 2-phenethylamine in three of the samples ranged from 88-111% (Table 3.2).

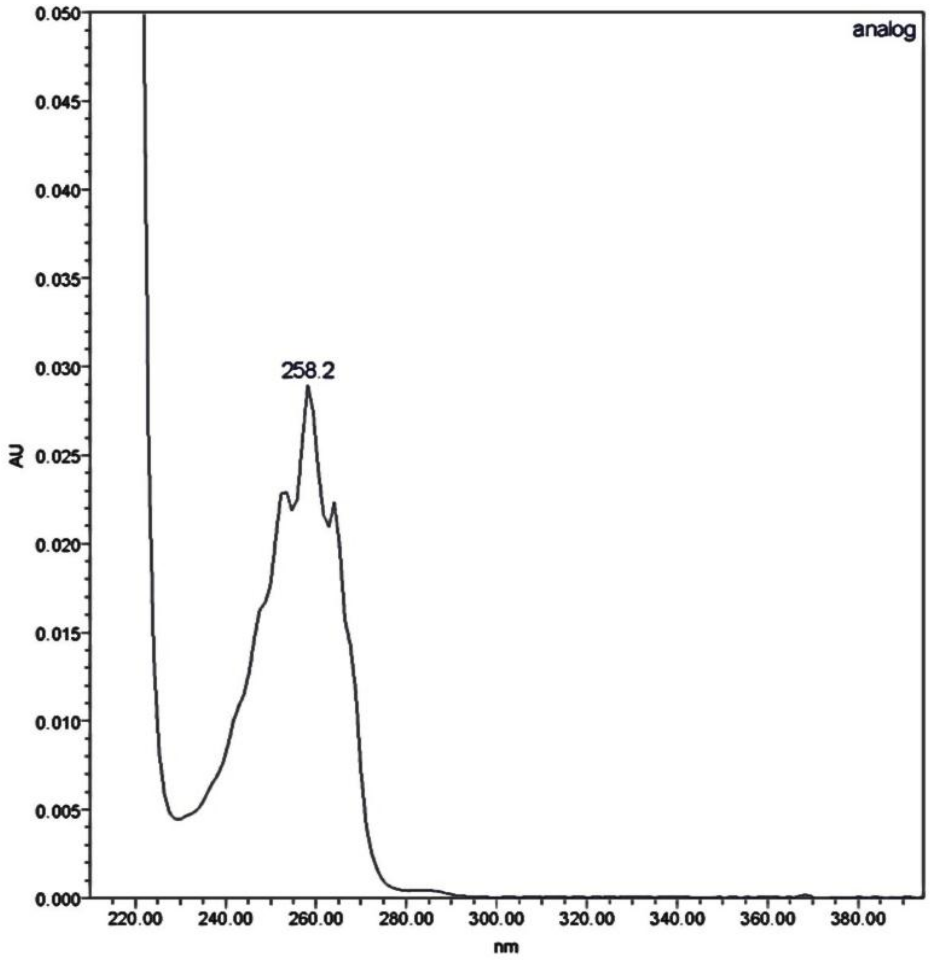

Figure 3.2. UV spectrum of N-ethyl-α-ethylphenethylamine

Table 3.2. Assay values and recoveries for phenethylamines in various samples.

Sample	Serving size	N-ethyl-α-ethylphenethylamine	2-phenethylamine	Recovery
A	5.8 g	3.4 ± 0.5 mg/g	12 ± 4 mg/g	105%
B	5.3 g	3.6 ± 0.2 mg/g	12 ± 6 mg/g	NA
C	5.8 g	4.2 ± 0.2 mg/g	11 ± 1 mg/g	88.1%
D	5.8 g	4.3 ± 0.5 mg/g	12 ± 3 mg/g	NA
E	5.3 g	4.3 ± 1.2 mg/g	12 ± 3 mg/g	NA
F	5.2 g	4.1 ± 0.3 mg/g	12 ± 2 mg/g	111%

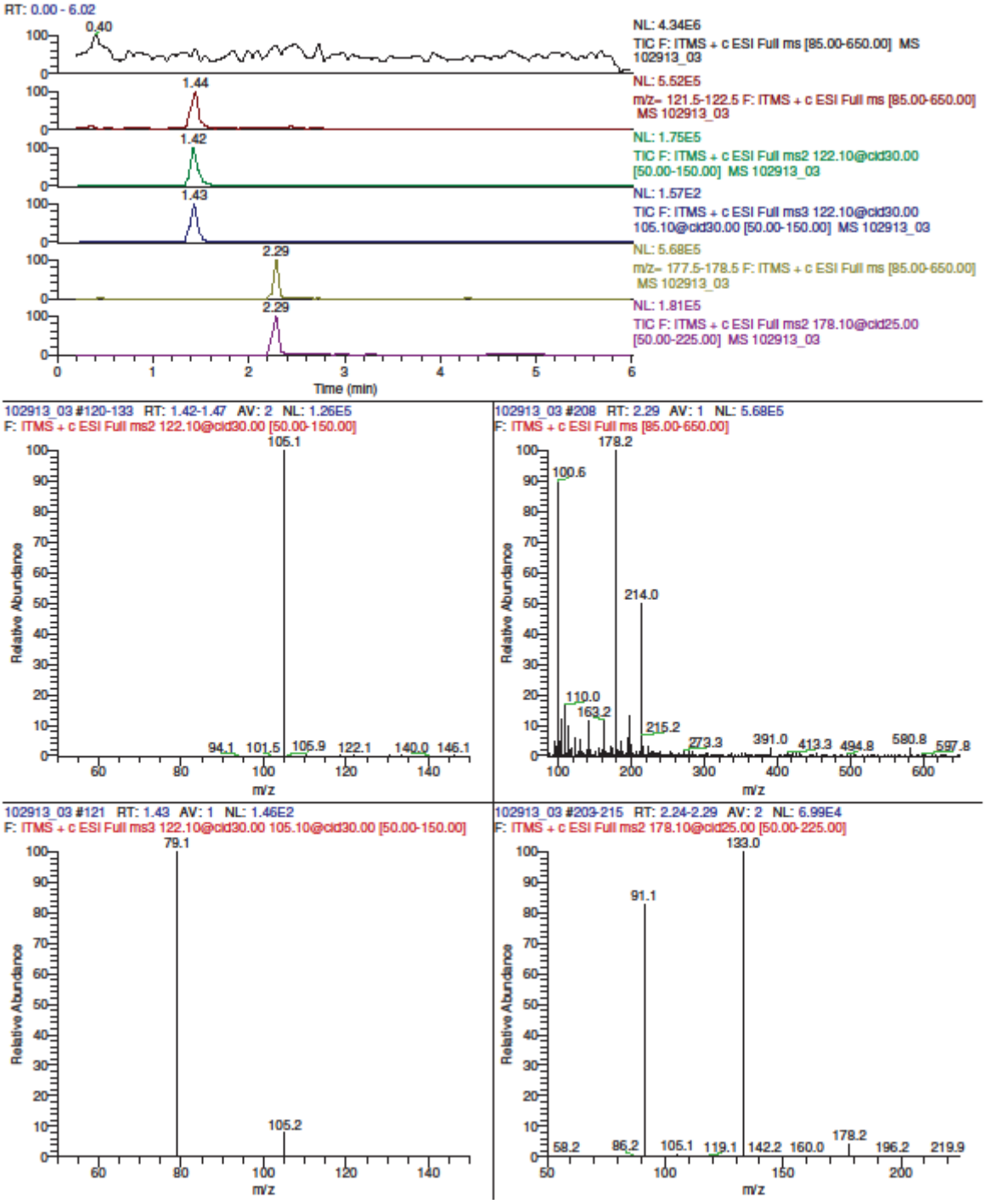

Figure 3.3. LC-MS data for phenethylamine analog

3.3.2 LC-MS Analysis

Accurate mass analysis of Samples A and B determined the *protonated* molecular formula of the analog to be $C_{12}H_{20}N$ based on a *m/z* of 178.15903 with an error of 0.02643 ppm for Sample A, and a *m/z* of 178.15887 with an error of -0.89095 ppm for Sample B. This is consistent with the value of 178.1581 for [M+1] from the literature and correlates to a molecular formula of $C_{12}H_{20}N$, and molecular weight of 177.291 g/mol, for the analog.

Using liquid chromatography mass spectrometry, two peaks were observed in the Samples A and B (Figure 3.3). The first peak, 2-phenethylamine, gave a retention time of 1.4 minutes and a full scan mass spectrum with an ion at *m/z* 122 $[M+H]^+$. In the MS^3 spectrum (122→105), there was an ion at m/z 79. Additionally, the analog gave a peak with a retention time of 2.3 minutes, and a full scan mass spectrum with an ion at *m/z* 178 $[M+H]^+$. In the MS/MS spectra, there were ions at m/z 133 and 91. The LC-MS data for the analog were consistent with the published literature values for this compound [5].

3.3.3 GC-MS Analysis

Analysis of the drink mixes yielded two peaks in the underivatized total ion chromatogram (TIC). One peak, at 9.5 minutes, had a spectrum consistent with the presence of a substituted phenethylamine, based on comparison of the spectrum to those of related compounds in a MS library, and *m/z* of 58, 91 and 148 (Figure 3.4) which are consistent with N-ethyl-α-ethylphenethylamine [5]. The most dominant ion, *m/z* of 86, suggests a $C_5H_{12}N$ group attached to the phenyl ring and the *m/z* of 176

suggests loss of hydrogen from the nitrogen to give a molecular weight of 177. A second peak in the underivatized TIC, at 15.0 minutes, had a spectrum consistent with

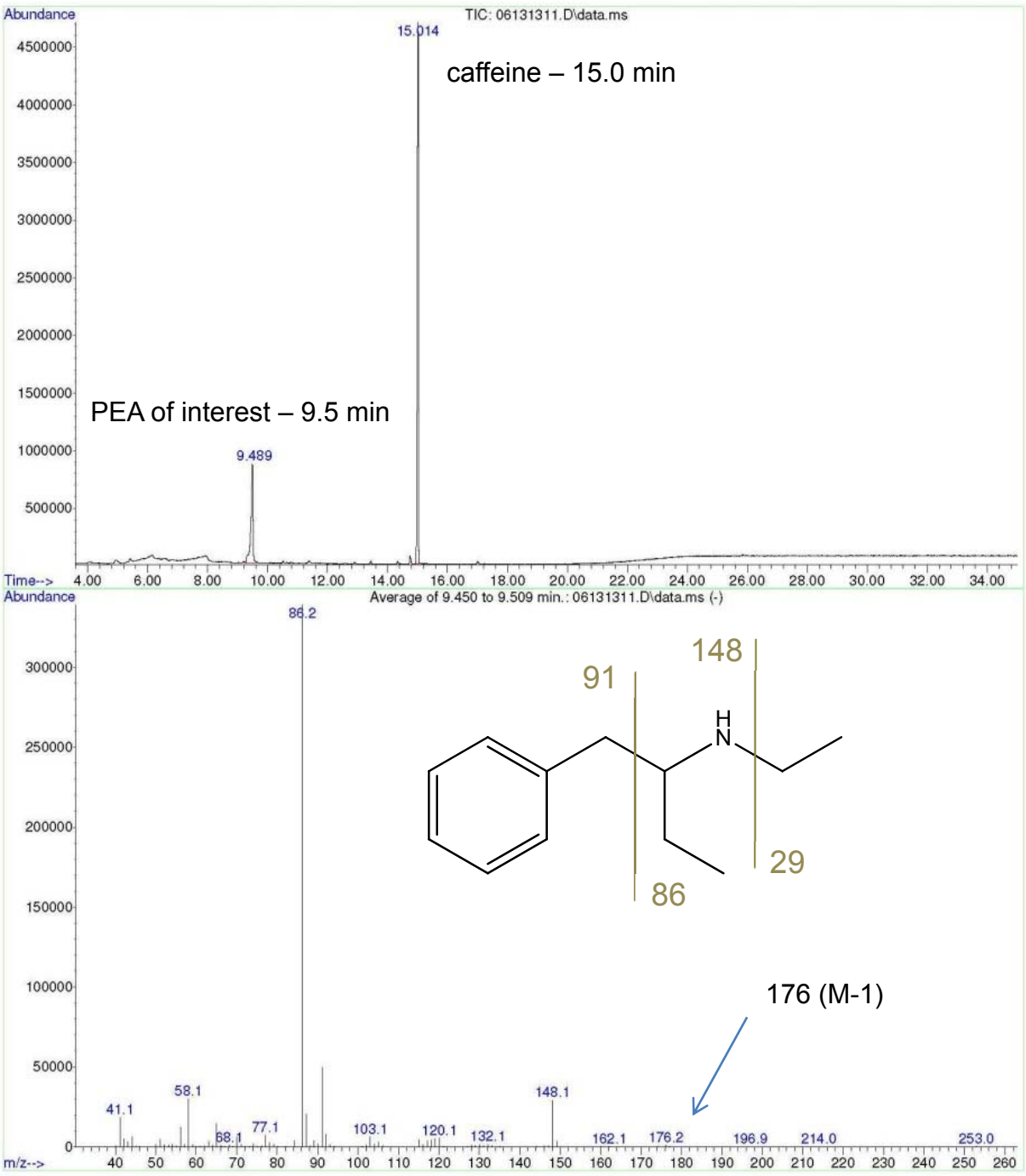

Figure 3.4. TIC chromatogram and mass spectrum of peak of interest.

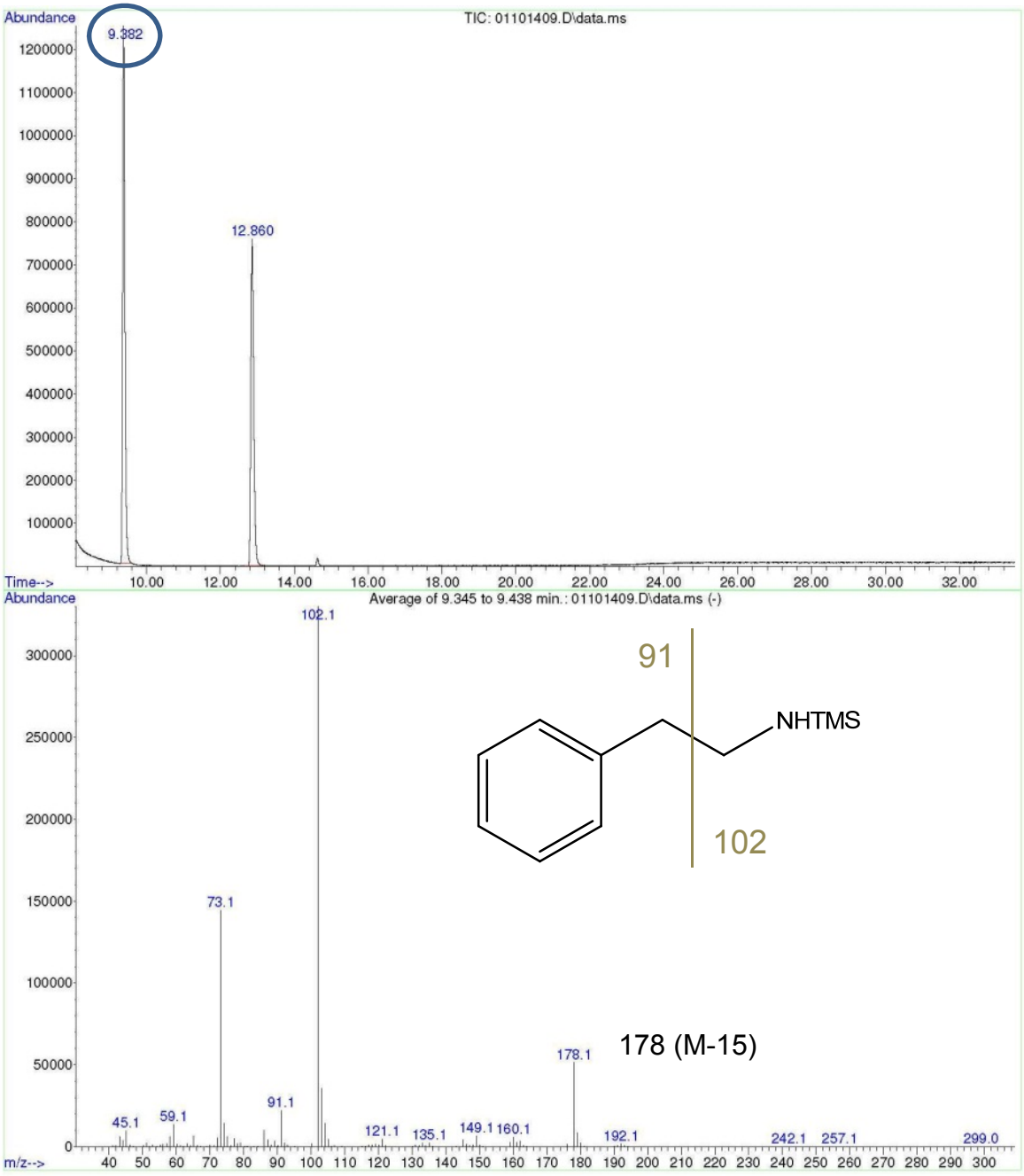

Figure 3.5a. TIC chromatogram and mass spectra of monoTMS 2-phenethylamine.

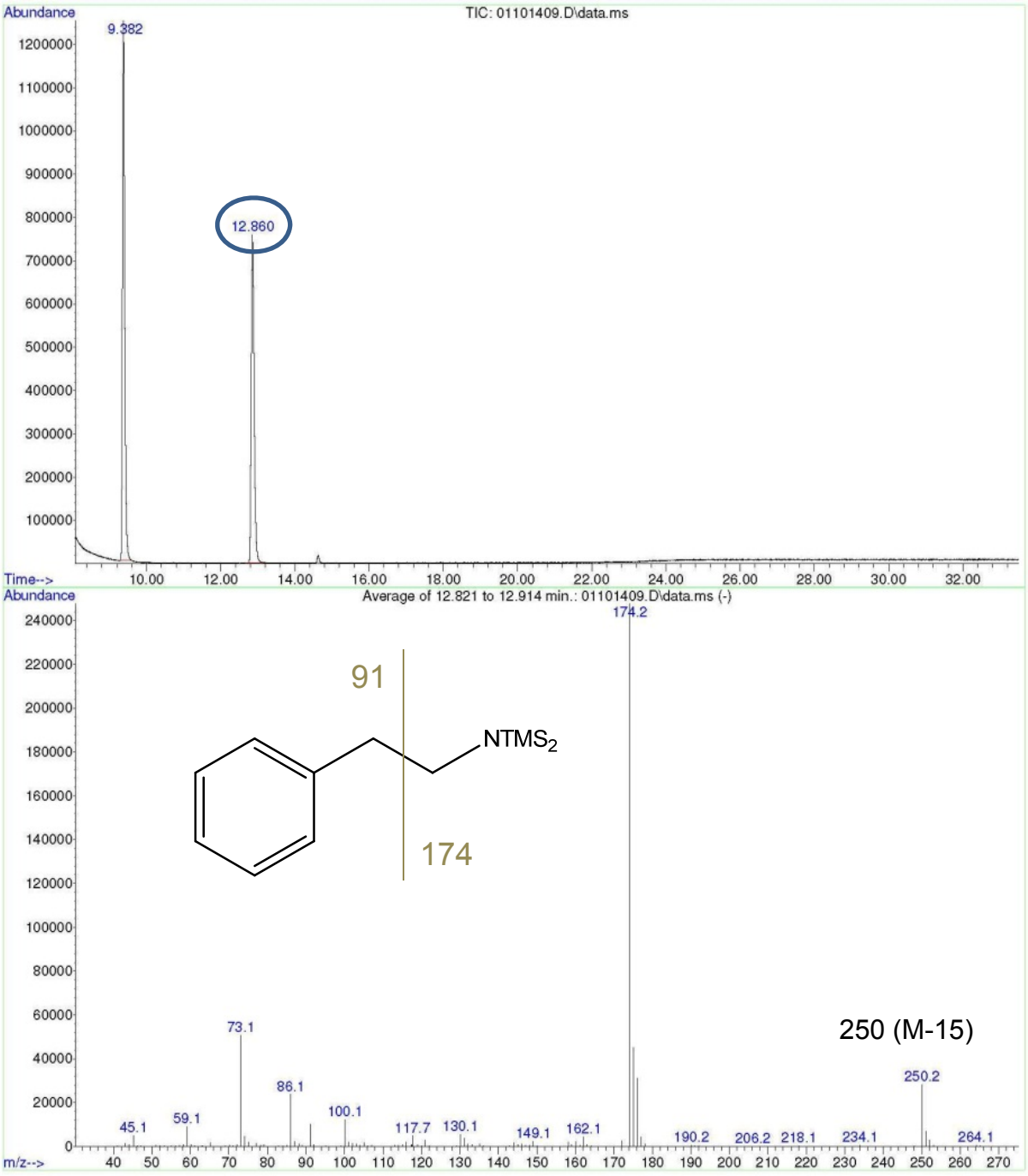

Figure 3.5b. TIC chromatogram and mass spectra of diTMS 2-phenethylamine.

the presence of caffeine, based on comparison of the spectrum to that of a MS library.

In order to increase volatility of components in the extract, derivatization is performed. In this case, the use of BSTFA causes active hydrogens (such as –OH, –SH and –NH) on a molecule to be replaced with a trimethylsilyl (TMS) group. Additionally, the primary amine of 2-phenethylamine is more responsive to derivatization, versus the hindered structure of a secondary amine, such as the one of N-ethyl-α-ethylphenethylamine, which may not form a derivative as easily. Generally, silyl derivatives are more volatile, less polar and more thermally stable, which makes them more amenable to GC-MS analysis [27].

The two peaks observed in the TMS derivatized TIC (Figure 3.5) have spectra consistent with the mono and di TMS forms of 2-phenethylamine, based on the fragmentation of the phenyl ring giving the largest ion at *m/z* of 102 and 174, respectively. Both caffeine and 2-phenethylamine were later confirmed based on retention time and mass spectral match to respective standards.

3.3.4 NMR Analysis

The 1D and 2D NMR spectra of the isolated fraction exhibited signals that were consistent with the published literature values [5] for this compound. Some minor variations in chemical shifts (Table 3.3) were observed versus the literature values, however all NMR correlations and multiplicities observed corresponded with the proposed structure. Specifically (Figure 3.6a), in the ^{1}H NRM spectrum, two methyl signals were observed at δ 1.32 and 0.98 (H-12 and H13) and the aromatic protons at δ 7.20–7.34 (H-6 through H-10). Using the 13C NMR spectrum (Figure 3.6b), 12 carbons were observed for the analog. Using 1H-1H correlation spectroscopy (COSY), the

spectrum of the analog showed two ethyl groups (Figure 3.6c). One of these groups, the H-12 methyl coupled to H-11, showed no correlation to any other protons, suggesting substitution to the nitrogen. The second ethyl group, H-12 methyl coupled to H-11, also showed correlation to H-2, because H-2 was coupled to H-3 and H-4. This suggests the ethyl group was connected to the C-2 carbon. Finally, the two nonequivalent H-4 protons were coupled to themselves, and H-2, further confirming the location of the ethyl groups and the resulting structure.

Table 3.3. NMR Chemical Shift Assignments (δ, ppm)

^{1}H NMR		^{13}C NMR	
Atom	δ (ppm)	Atom	δ (ppm)
H-1	9.20	C-2	60.3
H-2	3.31	C-3	22.6
H-3	1.70	C-4	36.3
H-4	3.17/2.89	C-5	136.4
H-6—H-10	7.20—7.34	C-6, C-10	129.3
H-11	3.03	C-7, C-9	129.0
H-12	1.32	C-8	127.2
H-13	0.98	C-11	40.7
		C-12	11.2
		C-13	8.6

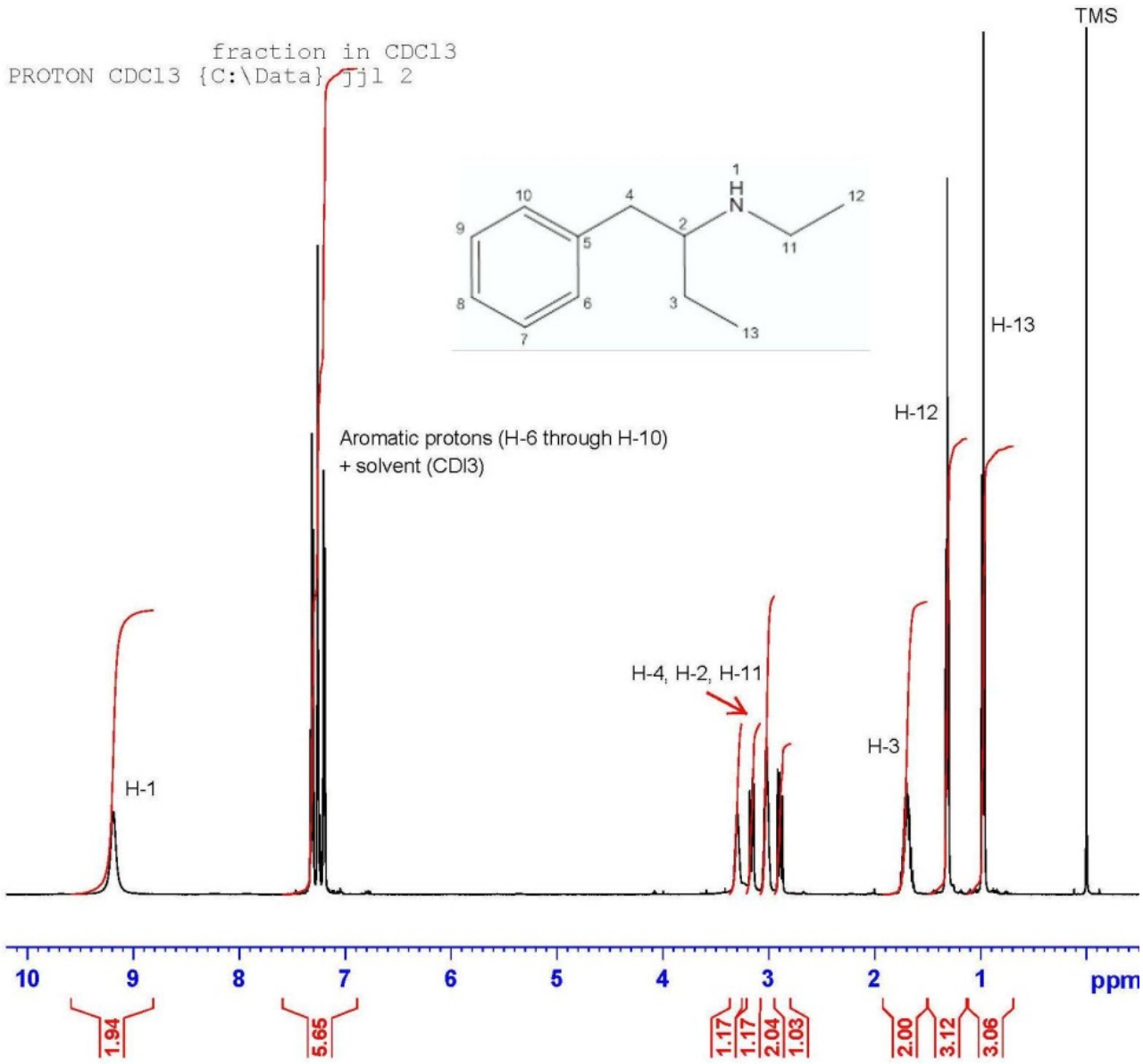

Figure 3.6a ^{1}H NMR spectrum of N-ethyl-α-ethylphenethylamine

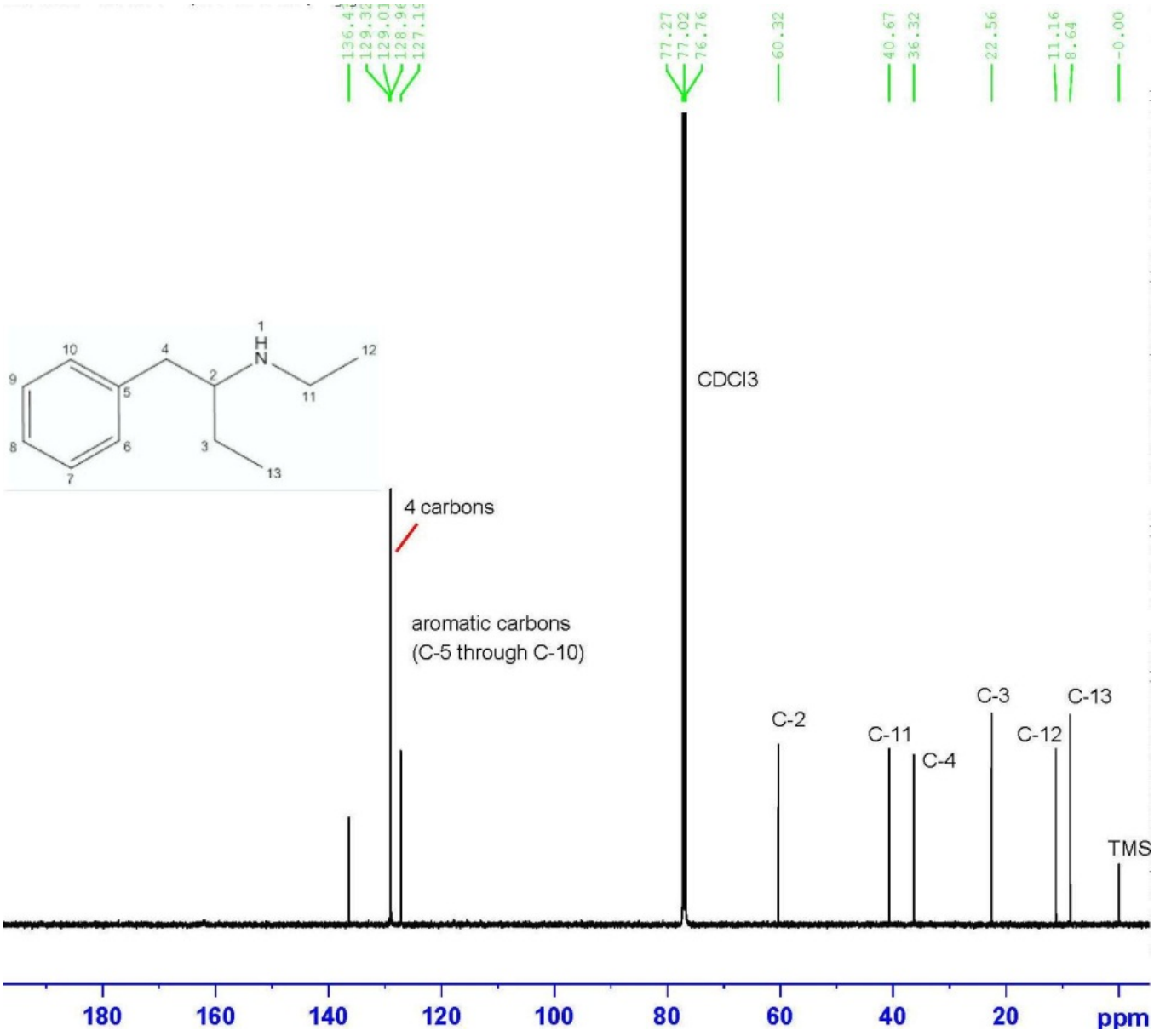

Figure 3.6b 13C NMR spectrum of N-ethyl-α-ethylphenethylamine

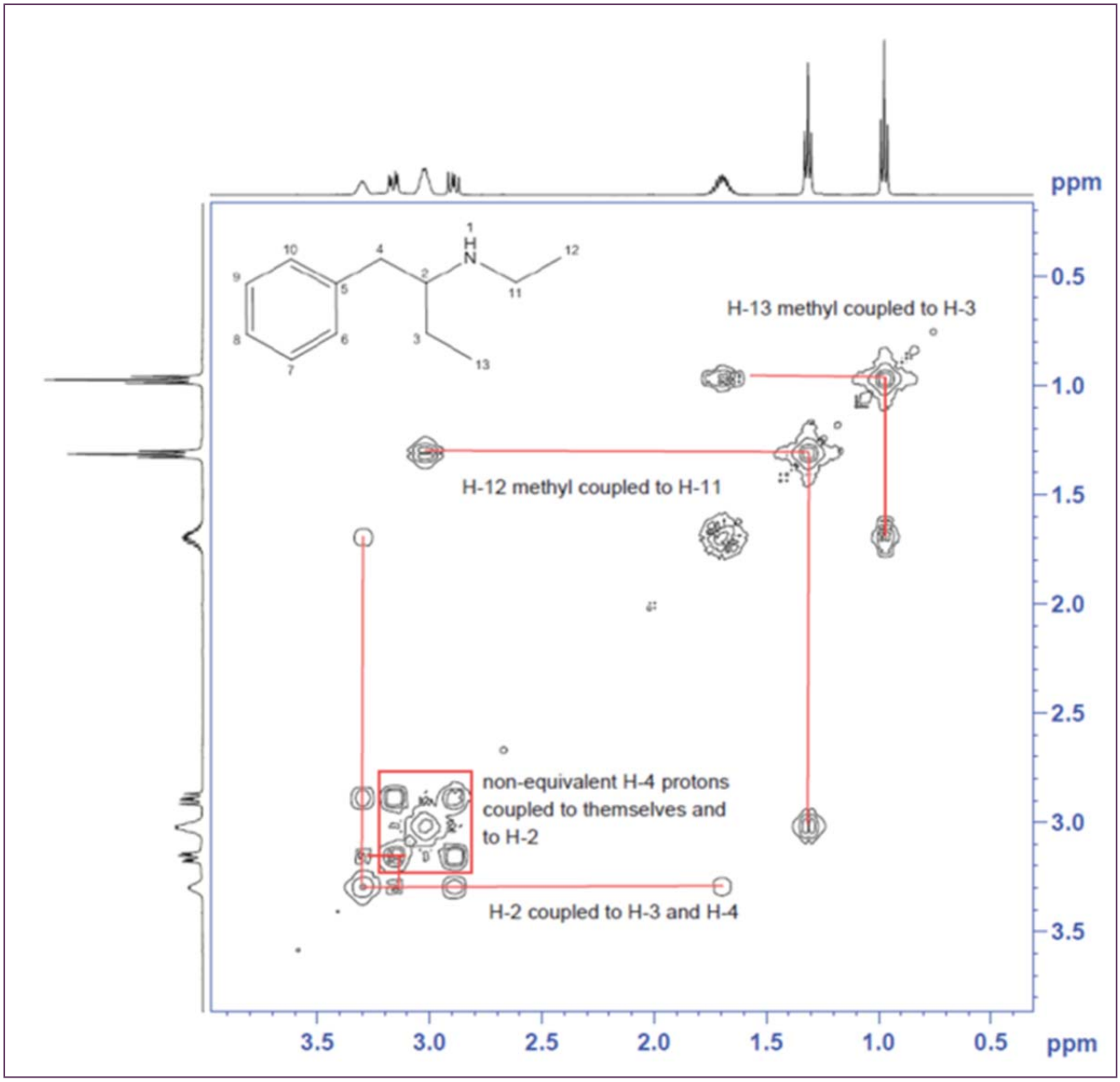

Figure 3.6c COSY spectrum of N-ethyl-α-ethylphenethylamine

3.3.5 Gradient Method

Five related compounds (Figure 3.7) were chosen for creation of an inclusive gradient method. First, 2-phenethylamine, $C_8H_{11}N$, was chosen to represent the base structure and lower molecular weight range at 211.30 g/mol. N,N-dimethylphenethylamine, (±)-methamphetamine and phentermine were chosen because they are all isomers of $C_{10}H_{15}N$, and the molecular weight of 149.23 g is in the middle range of substituted phenethylamines. N-ethyl-α-ethylphenethylamine was chosen because of its recent discovery in high priority samples, and it represents a higher molecular weight at 177.29 g/mol.

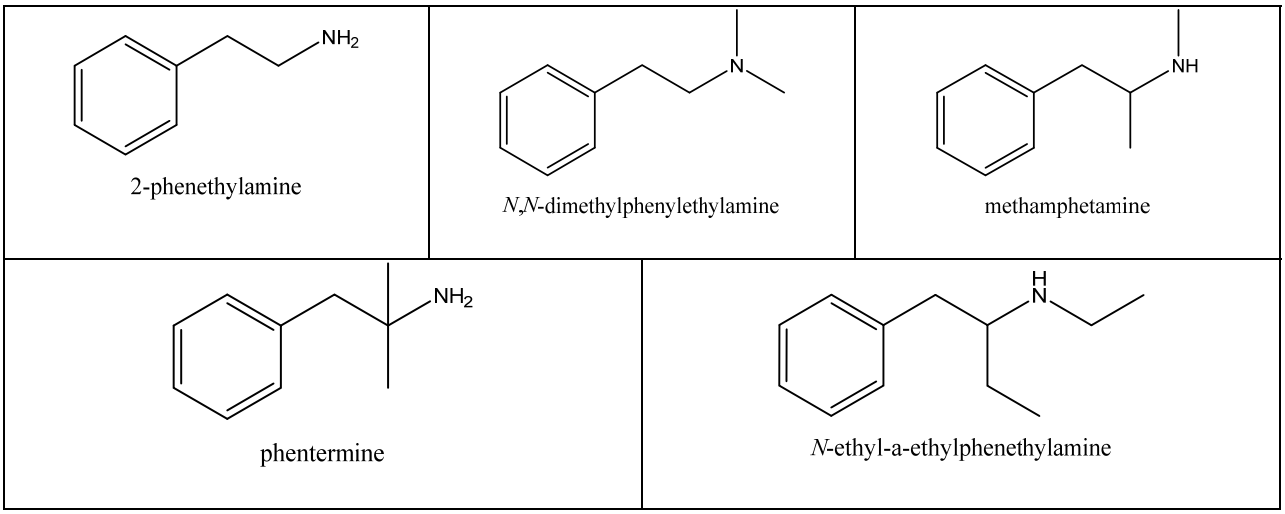

Figure 3.7. Structures of the five substituted phenethylamines used in the development of the gradient method.

The first peak to elute was 2-phenethylamine, followed closely by N,N-dimethylphenethylamine. The initial gradient parameters were highly aqueous in order to resolve this pair. Third and fourth to elute were methamphetamine and phentermine, respectively, followed by N-ethyl-α-ethylphenethylamine (Figure 3.8). The mobile phase at the end of the run was 90% organic. The compounds appeared to have eluted in

order of increasing molecular weight. Additionally, the three isomers eluted in order of increasing substitution on the C-2 carbon, and decreasing substitution of the amine group. The sodium phosphate buffer was a pH of 3.0, suggesting all amines were protonated.

Each calibration curve consisted of five standard concentrations made from a stock solution and serial dilutions. Concentration values for the free base of each standard ranged from approximately 1 mg/mL to 0.05 mg/mL, and were analyzed as a replicate of five injections. The variation for inter-day retention time data for all injections ranged from 0.3 to 1.6% RSD. The variation for inter-day for peak area data for five replicate injections over the five concentrations ranged from 0.038 to 4.5% RSD.

3.4 Conclusion

In this paper, a novel substituted phenethylamine was isolated from a complex pre-workout drink matrix marketed as a dietary supplement and characterized using HPLC-UV, GC-MS, NMR and accurate mass MS. This data was consistent with a crystalline powder seized by narcotics agents in South Korea and identified as N-ethyl-α-ethylphenethylamine. It is likely to be related to the pharmacokinetics of methamphetamine, and should be monitored closely and screened for in samples suspected of eliciting euphoric effects.

Additionally, this paper described a method that provides separation of five substituted phenethylamines in fifteen minutes. It is linear over a wide range of concentrations and sensitive to trace levels. UV data presented indicated that the substituted phenethylamines have similar spectra, and this can be useful in the characterization of any new related compounds discovered.

As additional analogs of phenethylamine are created, it may be difficult to stay up to date on the characterization of the structures and any related contaminants. It will be important to use a wide range of analytical techniques to identify the unknowns, and follow up with quantification of any compounds detected, especially as information related to dosage, side effects and toxicity becomes available.

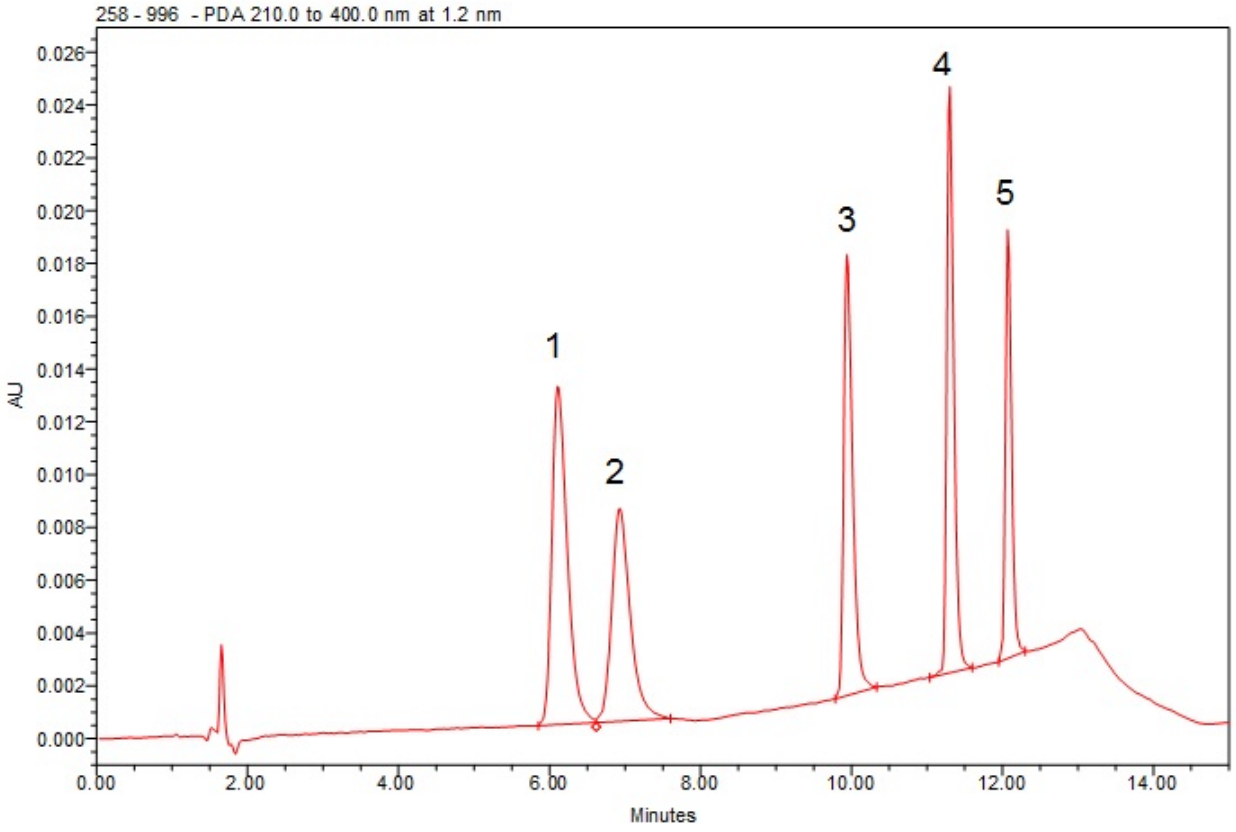

Figure 3.8. Chromatogram of phenethylamine and related compounds. (1) 2-phenethylamine, (2) N,N-dimethylphenethylamine, (3) methamphetamine, (4) phentermine and (5) N-ethyl-α-ethylphenethylamine. Concentration values for the free base were approximately 0.2 mg/mL for each compound.

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Chapter 4 – Overall Conclusion and Future Work

Determining the identity of illegal drugs and active pharmaceutical ingredients in various matrixes often involves a two prong approach in an analytical laboratory setting. It is especially important for any chemical identification to have a high level of certainty before reporting results to outside sources, such as regulatory organizations or the media. This can generally be assured with the use of two independent, scientifically uncorrelated techniques. At minimum, this often means the use of a chromatographic technique for a retention time match, and a spectrometric technique for structure elucidation. More accepted by the scientific community, especially for compounds that are relatively unique or new to the market, is the inclusion of two spectrometric techniques, such as a UV-Vis, mass spectrometry, or infrared spectroscopy.

For PDE-5 inhibitors, the GC/FT-IR/MS described is able to identify sildenafil, tadalafil and vardenafil successfully using chromatographic retention, mass spectral data and infrared spectra. MS data is capable of providing a molecular weight and information about the structure based on fragmentation patterns. IR data is able to provide information related to functional groups, saturation and stereochemistry. The analysis can be done quickly and simply on filtered sample dilutions, or the chromatography and sensitivity can be improved to values consistent with LC-MS by performing a TMS derivatization. Additionally, this method shows promise in being able to detect, differentiate and determine assay values, in the derivatized mode, for the numerous PDE-5 inhibitor analogs that have been detected in various samples.

The class of substituted phenethylamines can be identified by their absorbance spectra and can be accurately assayed using HPLC-UV, Additionally, they could further

be analyzed using GC/FT-IR/MS. In general, phenethylamines are quite amenable to derivatized GC/MS analysis, and the combination of two spectrometric detectors could identify the chemical structure. The sensitivity of IR could be used to determine the presence of functional groups and differentiate stereoisomers, and the mass spectral data could provide a molecular weight and fragmentation to show further characterize structure. Additionally, the use of chromatographic separation can take the place of extensive sample prep and analyte extraction.

Most analytical laboratories have access to GC-MS, FT-IR and HPLC-UV, as these instruments are easy to operate and don't generally require extensive maintenance. Using the designed protocols in the chapters described above, identification and quantification of PDE-5 inhibitors, substituted phenethylamines and other categories of drugs can be accurately determined with a high level of certainty. The merging of these techniques into one analysis, such as GC/FT-IR/MS (or FT-IR coupled to HPLC-UV) can save analyst time in the lab, conserve reagents and consumables, and also reduce the need for additional sample portions and preparations. Finally, the results collected are comparable to those determined from techniques such as LC-MS.