2021

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Spring 2020-Spring 2021

Date of Submission: May 11, 2021

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science at Virginia Commonwealth University.

Acknowledgements

I would like to thank everyone who played a significant role in my academic accomplishments. Firstly, I would like to express my utmost gratitude and appreciation to my research advisor Dr. Catherine Connon for the knowledgeable advice, comments, and endless support she has given me throughout this research project. Secondly, I would like to express my sincere gratitude to Brittany Hudson, to whom this research project would not have existed. Thirdly, I would also like to thank Dr. Joseph Turner and Dr. Christopher Ehrhardt for the assistance in the project as well. I would finally like to thank all of my friends and fellow classmates for their support and guidance.

Abstract

Blood is often a type of evidence found at crime scenes. There has been a long history of criminal cases involving blood evidence samples that were allegedly planted at the scene by a third party. Ethylenediaminetetraacetic acid, or EDTA, is an anticoagulant that is not naturally occurring in blood samples; it chelates to ions in blood to prevent clotting. If present in a blood evidence sample, it could be indicative that the blood sample may have been planted at the crime scene to implicate a suspect. EDTA is also reported to modify clothing and exist in detergents, to allow dyes to set and prevent rancidity in soaps. Current techniques for the identification of EDTA include liquid chromatography-tandem mass spectrometry. But this can be an expensive, destructive, and time-consuming method of analysis. Spectroscopic methods like Raman spectroscopy and Fourier-transform infrared spectroscopy (FTIR) provide fast, non-destructive alternative methods to confirm the presence of EDTA in blood evidence. This research aimed to develop methods for both Raman and FTIR spectroscopy to allow for rapid identification of EDTA. FTIR parameters including the number of scans, optical velocity, and resolution were experimented with, to identify the most sensitive method for the identification of EDTA. The optical velocity and resolution were reduced to attempt to increase the sensitivity to EDTA. The number of scans was increased for the same reason. The developed FTIR and Raman spectroscopic methods were suitable for detecting EDTA in water-based standards at concentrations as low as 1.5 mg/mL. EDTA can be observed with peaks reported at approximately 1615 cm^{-1} and 1401 cm^{-1}. However, these methods were not suitable for confirming the presence of EDTA in blood under normal, expected concentrations of 1-1.5 mg/mL. When analyzing the samples using FTIR, the amide I and II absorption bands present in blood prevented the EDTA bands of interest from being confirmed. Future paths include investigating other anticoagulants like potassium EDTA, heparin, and sodium citrate, and determining whether the amide I and II bands from blood evidence samples can be removed while isolating the EDTA within the sample.

Keywords: forensic science, blood, ethylenediaminetetraacetic acid, Raman spectroscopy, Fourier-transform infrared spectroscopy

Introduction

Within the last several decades, there has been a long history of criminal cases involving alleged planting of blood evidence by a third party. This type of evidence has been placed in the spotlight due to highly publicized cases like the O.J Simpson trials [1]. Ethylenediaminetetraacetic acid, or EDTA, is an anticoagulant that when mixed with blood, will form metal complexes with calcium and magnesium ions in the blood [2]. EDTA will chelate any ions in the blood but has the strongest affinity to magnesium ions. The chelation of the metal ions to form an EDTA-metal ion complex is what prevents blood from clotting. Figure 1 demonstrates how EDTA chelates around a magnesium or calcium ion to form the complex. EDTA is not a natural component of blood; if it is detected in a sample, it may indicate that the blood sample originated elsewhere and was planted at the crime scene. EDTA is also present in certain detergents and soaps, which could also cause EDTA to be associated with a blood evidence sample. EDTA is added to soaps to prevent rancidity; the concentration of EDTA in these types of products varies from 0.05% to 0.25% [3]. When washed, the detergents may leave trace amounts on clothing. EDTA is also used to improve the aggregation of dye onto fabrics like cotton; it neutralizes the negative charge potential that cotton possesses [4]. Therefore, EDTA present in a blood sample may be a result of how the substrate was processed during manufacturing.

The **R**everse **E**DTA **D**etection in **Bl**ood using **E**BT and **U**V-Vis test (RED-BLEU) is currently being developed and validated by our laboratory as a presumptive method for the detection of EDTA in blood samples [5, 6]. To date, this is the only presumptive test established for the detection of EDTA in blood samples that is noted in the literature. The RED-BLEU method is a colorimetric test that uses a complexometric indicator, Eriochrome® Black T (EBT),

to produce a blue color when EDTA is present and a pink color when not present. The mechanism driving the test is that the EDTA-calcium and EDTA-magnesium complexes formed are stronger than those produced between these ions and the EBT indicator [6]. Thus, when EBT is added to the sample it will remain unbound, yielding the visible blue color of the indicator. Blood samples that do not contain EDTA will yield a pink color change as the indicator actively binds to the ions and undergoes a conformational change.

The UV-Vis portion of the RED-BLEU assay is designed to supplement the colorimetric titration to assist with color interpretation, eliminate some false positives, and estimate the amount of EDTA detected [5]. For this portion of the assay, an absorbance peak at ~192-196 nm is indicative of EDTA being present (blue) and is used to estimate EDTA concentration. On the other hand, samples without EDTA (pink) lack this ~192-196 nm peak and instead exhibit a weak, broad peak from ~500-700 nm with a point of inflection around 590nm. Samples that are visibly blue are noted as false positives when they lack both the EDTA peak and the weak, broad peak at ~500-700 nm. The assay is sensitive to the presence of EDTA in a sample with a reported limit of detection of 0.1 mg/mL, but it is only a presumptive test [5]. In addition, the 192-196 nm peak may be difficult to observe at times because it is at the edge of the ultraviolet-visible light range, and is also due to blood [5].

Confirming the presence of EDTA in blood samples using spectroscopic methods for forensic analysis is an area that has not been widely investigated. The original work regarding EDTA detection in blood involves the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS), which can be expensive and require significant sample preparation [7,8]. Spectroscopic methods may be utilized instead of chromatographic methods due to their nondestructive nature, relatively low cost, and compatibility with a mobile laboratory. Both

Raman spectroscopy and Fourier-transform infrared spectroscopy (FTIR) are common spectroscopic techniques utilized in forensic science that have high sensitivity, are non-destructive, and have mobile adaptations. FTIR and Raman spectroscopy may be suitable methods for EDTA identification, because they measure the absorption patterns of chemical structures to produce spectra for that sample, that acts as a molecular fingerprint for that compound. The absorption patterns are characterized based on the types of bonds present in the sample (i.e., FTIR), and/or the types of vibrations or stretching that those bonds exhibit (i.e., Raman Spectroscopy). EDTA exhibits both asymmetric and symmetric vibrations with its CO_2 bonds, as well as COO^- bonds associated with carboxylic acid groups.

Raman spectroscopy is a "vibrational spectroscopy" technique, complementary to infrared spectroscopy [9]. Raman utilizes a light source, typically a laser beam, to produce an inelastic light scattering event [9]. The light scattering can occur as either 'Rayleigh', 'Stokes', or 'Anti-Stokes scattering' [10]. In Rayleigh scattering, the photons have the same energy level as the incoming radiation from the laser. This is termed elastic scattering [9, 10]. The inelastic scattering occurs in Stokes and Anti-Stokes scattering, where the photons have lower energy and higher energy than the incident radiation, respectively [9, 10]. Raman spectroscopy is extremely sensitive to symmetric covalent bonds present within the compound of interest [10]. The scattering is fully dependent on the composition of the compound and how the incident radiation interacts with the molecules, thus producing unique spectra for each analyte analyzed. Few publications have been produced regarding the detection of EDTA using Raman, and none have been done with regards to forensic analysis of EDTA. One of the publications involves using Surface-Enhanced Raman Scattering (SERS) of the EDTA-disodium salt complex, but only on silver electrodes [11]. In this case, the wavenumbers associated with the EDTA complex were

913 cm^{-1}, 935 cm^{-1}, and 1408 cm^{-1}, respectively [11]. Expected locations of the absorption bands produced from EDTA present in dried blood samples were reported at approximately 1100 cm^{-1}, 1200 cm^{-1}, and 1300-1400 cm^{-1} [12].

Raman Spectroscopy has only recently been used to presumptively detect blood and other body fluids in a forensic context. It has been used to presumptively differentiate animal species to assist in differentiating animal samples from human blood samples [13]. Blood originating from different species will absorb energy from the laser source at varying levels. The laser radiation was discovered to be nondestructive to the samples, which is an added bonus over other methods performed (like HPLC-MS), which may include sample preparations involving derivatizations [12]. It also requires little to no sample preparation; the light source can typically be placed directly onto the sample [14].

An FTIR instrument functions by passing an infrared beam through the analyte of interest. Some of the infrared radiation will be absorbed by the sample, while some will transmit through the sample. With FTIR-ATR, or attenuated total reflectance FTIR, the infrared light does not get absorbed by the sample. Instead, the IR beam passes through the ATR crystal, reflects back, and produces an “evanescent wave” that interacts with the sample. The amount of infrared radiation is absorbed is dependent on the molecular composition of the sample. The output signal is the result of the absorption/transmission pattern, and results in a spectra that is characteristic of that sample. Similar to Raman spectroscopy, an incident radiation is used to generate vibrational frequencies, which are unique to the compound of interest [9]. However, FTIR is much more sensitive to dipole moments, where the electrons are not shared equally, rather than symmetrical covalent bonds [9]. FTIR is also much more sensitive to interference from water, which is why Raman may be better suited for body fluid characterizations [9]. FTIR analysis provides

orthogonal information regarding the analyte composition, which is why it should be used in conjunction with Raman if possible, especially regarding detecting the presence of EDTA in blood samples.

Blood has been analyzed using spectroscopic methods. FTIR-ATR has been used to discriminate between animal and human blood [15]. Eleven animal species (cat, dog, rabbit, horse, cow, pig, opossum, raccoon, deer, elk, and ferret) were analyzed using FTIR-ATR and compared to a human blood sample. The spectral ranges were the same for all of the samples, but the ATR transmission percentage varied for each species. The species differences were due to changes in protein, lipid, and nucleic acid composition. However, no specific dissimilarities in the blood composition between the different species were identified [15]. Human blood samples produced a FTIR-ATR spectra with the lowest transmission percentage, while ferret blood samples produced spectra with the highest transmission percentage [15]. However, little research has been established regarding the detection of EDTA in blood using FTIR spectroscopy. The spectroscopic characteristics of blood have been identified [16]. In spectra of whole blood, the vibrations of the OH bond presents a broad peak at around 3400 cm^{-1}. In addition, there are abundant amide I and amide II bands that are commonly present due to proteins in biological materials. The wavenumbers of the amides are about 1641 cm^{-1} and 1539 cm^{-1}, respectively [17].

EDTA has been analyzed using FTIR-ATR, but the majority of the research is in the medical field, regarding the metal complexation produced by various anticoagulants, and how it affects blood samples and testing. The chelating properties of EDTA in blood are known to alter medical testing. For example, fluorometric methods are used to quantify hormonal levels in blood. When EDTA is present, it can result in the hormonal levels being falsely reported [18]. Another medical study involved identifying the effects that EDTA has on blood samples that

have been stored for a prolonged amount of time prior to testing. The results showed that when blood samples with EDTA are stored for at least four days prior to any testing, the EDTA reacts with the blood and can change the morphology of the red blood cells, as well as increase their fragility [19]. When using FTIR-ATR to analyze EDTA, the common vibrational bands reported for disodium EDTA are at 1619 cm^{-1}, 1402 cm^{-1}, 1358 cm^{-1}, and 1324 cm cm^{-1}, when a 0.1 M (37.22 mg/mL) EDTA solution in water was analyzed [9]. These are indicative of asymmetric CO_2 vibrations, symmetric CO_2 vibrations, NH^+ stretching, and the COO^- bond (affiliated with the carboxylic acid group), respectively [17]. When performing spectroscopic analyses for the detection of EDTA, the aforementioned bands are the major bands of interest.

The specific aims of this study were to initially determine what absorption bands are unique to EDTA when using spectroscopic methods such as FTIR and Raman spectroscopy. Next, if detected, confirmatory tests would be developed for the identification of EDTA in a blood evidence sample. The proposed confirmatory tests would determine the detection limit of EDTA, and the minimum sample size required to detect EDTA.

Materials and Methods

Standard Preparation

Initially, the EDTA standards were prepared using solid disodium EDTA. Due to the issues dissolving the EDTA and the variation in the absorbance intensity of the IR bands, the EDTA standard solutions were prepared using a 0.1 M standard solution of EDTA in water (Fisher, Lot No. 89133.776). The standard solution was then used to prepare a 30 mg/mL EDTA stock solution. From there, a serial dilution was performed to prepare the standard solutions at

concentrations of 10 mg/mL, 6 mg/L, 3 mg/m, 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.05 mg/mL, respectively. These concentrations were chosen to establish a suitable calibration around the typical concentration of EDTA in blood, which is 1-1.5 mg/mL,. It is also anticipated that blood evidence samples may be diluted. At a crime scene, a suspect may try to wash away the blood present, which would dilute any remaining sample. The location of the crime scene is also a factor; blood samples may be exposed to other elements like the weather, and be partially washed away. It is also reasonable that blood containing EDTA could be deposited on an existing, authentic blood stain, hence the need to use blood without EDTA as a diluent for the blood with EDTA sample. Thus, the concentrations chosen for the standards anticipated possible dilution concentrations. HPLC grade water was used to prepare the standards (Fisher, Lot No. 188693).

The human blood sample used for this project was purchased from BioIVT (Westbury, NY) and contained disodium EDTA with 1.5 mg/mL EDTA (Lot No. HMN87767). The blood sample was then prepared via serial dilution to obtain the following concentrations: 0.75 mg/mL, 0.3 mg/mL, 0.15 mg/mL, 0.075 mg/mL, 0.05 mg/mL, and 0.03 mg/mL. Blood was diluted to 0.75 mg/mL, 0.3 mg/mL and 0.15 mg/mL using both blood and water as two separate diluents; water was used as the diluent for the EDTA standards that were prepared using both the solid disodium EDTA and the liquid 0.1 M EDTA stock solution.

FTIR Spectroscopic Methods

The goal of the spectroscopic analyses was to isolate absorption bands associated with the EDTA from the absorption bands associated with blood samples, incorporating good signal to noise ratios and background correction, and identify the detection limit of the anticoagulant. The signal to noise ratios would be calculated for each concentration where the EDTA was

detected, and linear regressions would be performed to validate the relationship between the intensity of the EDTA bands and the concentration of the EDTA in the samples.

A Nicolet iS50 FT-IR spectrometer in combination with the corresponding OMNIC software was used to view and analyze the data. For the FTIR analysis, all samples (EDTA standards and blood with/without EDTA) were examined in liquid form using 20 µL pipetted directly onto the ATR crystal and processed five times each. Spectra were collected immediately for EDTA standards, while blood samples were allowed to set for 30 sec following deposit onto the ATR crystal; all spectra collected were analyzed in common scale. It is noteworthy to mention that whole blood without EDTA was collected from a single donor and deposited within 5 min of collection. This served as the background for the blood dilutions with EDTA. Additionally, a sample size of 20 µL was necessary, because as the method was being developed, the EDTA standards evaporated off of the sample area of the instrument.

Optimization of FTIR Parameters

The absorption bands present at 1619 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1}, and 1322 cm^{-1} are the peaks of interest used to identify whether the EDTA was detectable in the standards and blood samples. These correspond to asymmetric CO_2 vibrations, symmetric CO_2 vibrations, stretching associated with the NH^+ bond, and the bonds associated with the carboxylic acid groups [7]. The default FTIR settings were tested first, using a DTGS ATR detector. Therefore, 32 scans were collected with a 4 cm^{-1} resolution, an aperture of 100 was used, the optical velocity was set to 0.4747, and the wavenumber range was 4000-350 cm^{-1}. The number of scans alters the result of the average spectra. The more scans taken of the sample, the smoother the spectra will be, due to an increase in the signal-to-noise ratio. To try to increase the sensitivity of the instrument and lower the detection limit of EDTA, the number of scans was initially increased to 64. In addition,

the other parameters including the aperture, optical velocity, level of attenuation and resolution were changed. These parameters were repeatedly tested in various combinations to try to determine a way to confirm the presence of EDTA in samples in concentrations expected to be in blood evidence samples. The aperture was set at values of 87 and 100. The optical velocity was set to 0.3165, 0.4747, and 0.6235 to identify which setting was the most suitable. The level of attenuation was set to either none, or moderate. The resolution settings used were 2 cm^{-1}, 4 cm^{-1}, 8 cm^{-1}, and 16 cm^{-1}. The resulting collection parameters for the EDTA standards was as follows: 64 scans, 4 cm^{-1} resolution, aperture of 100, optical velocity of 0.4747, and range of 4000-350 cm^{-1}.

Statistical Analysis of FTIR Results

Once the method was developed for the spectra with the highest absorbance intensities at the 1615 cm^{-1} and 1401 cm^{-1} peaks, resulting in suitable signal to noise ratios, five spectra were collected for each of the EDTA standard solutions. Initially, the sample spectra observed were analyzed with the default full scale option. Full scale normalizes the spectra around the tallest peak. The result is a spectrum that is not properly normalized. Common scale normalizes the data respective to each other, which is useful in accurately comparing the band intensities of various sample spectra. All resulting spectra were then formatted to common scale, especially those of the standard solution replicates. For each of the replicates, the absorbance values for the peaks of interest at 1615 cm^{-1} and 1401 cm^{-1} were recorded. The average absorbance values for the 1615 cm^{-1} peak were then plotted against the concentration of the EDTA standard, to make a linear regression plot. The goal of the linear regression was to identify if the samples were increasing in absorbance linearly with the concentration value. The signal to noise ratios were also calculated for both the 1615 cm^{-1} and 1401 cm^{-1} peaks.

Optimization of Substrates using FTIR

A substrate study was performed as part of the method development for FTIR. Cotton swabs, foam swabs, and filter paper were used as possible substrates for both the EDTA standards and the 1.5 mg/mL EDTA blood sample. The 30 mg/mL, 10 mg/mL, 6 mg/mL, and 3 mg/mL EDTA standard solutions were the standards deposited onto all of the substrates. The 30 mg/mL and 10 mg/mL standards were the two highest concentrations. However, the lower EDTA concentrations at 6 mg/mL and 3 mg/mL were also analyzed, to see how the absorbance values might change. The 1.5 mg/mL EDTA in blood sample was also deposited onto the substrates. For all of the substrates, 20 µL of the sample was deposited and left to air dry overnight. Both air and the substrate blank were subtracted out as the background, to identify which one was more suitable.

Method Development of Blood Samples Using FTIR

The liquid blood samples with EDTA were the last samples to be analyzed using FTIR. The 1.5 mg/mL EDTA in blood sample was run on the FTIR, where 20 µL of the blood was pipetted onto the ATR crystal. Water and whole blood without EDTA were tried as background subtractions. Various blood dilutions were analyzed, both as the sample and as the background subtraction. Finally, a blood sample was spiked to 30 mg/mL of EDTA to determine whether the EDTA could be identified at such a high concentration in a blood sample.

Raman Spectroscopic Methods

The objective of Raman spectroscopy experiments were to identify the detection limit of EDTA, and compare it to results obtained with FTIR. Furthermore, the goal was to determine

whether the EDTA could be detected in blood samples, and to see whether the glass slide was a suitable substrate for dried bloodstains. In anticipation of interference from fluorescence, a 780 nm laser was used. For sample preparation, 20 µL of the sample (for EDTA standards and blood with/without EDTA) was pipetted onto a glass slide and dried overnight at room temperature. Initial collection parameters included a wavenumber range of 3422-56 cm^{-1}, 2.4-4.4 cm^{-1} resolution, and 10 mW laser power. The laser power was reduced to below 10 mW, to determine whether that would help with the glass interference. The wavenumber range remained the same. The aperture was also decreased to less than 25 µm, to determine whether this would remove the interference from the glass slide.

Results & Discussion

FTIR

A whole blood sample without EDTA was analyzed on the FTIR, so that the peaks of the blood could be compared to samples with EDTA. Furthermore, the EDTA without blood spectrum would be used as the background subtraction for the blood samples with EDTA. The peaks of associated with the blood were observed at approximately 1650 cm^{-1} and 1544 cm^{-1} (Figure 2). These peaks correspond to the amide I and II bonds present in blood. For reference purposes, to try to identify peaks of interest, an FTIR spectra of solid disodium EDTA was collected, where 3.0 mg of solid EDTA was added onto the ATR crystal. Results are shown in Figure 3. Although shifted, the bands of interest are at around 1610 cm^{-1} and 1392 cm^{-1}. For the solid EDTA, air was subtracted out as the background spectra, which can be seen in Figure 4.

For the collection of the EDTA standards spectra, water was subtracted out as the background. Initially, the standards were prepared using disodium EDTA, which was then

dissolved in HPLC grade water and vortexed. It was observed that the EDTA did not readily dissolve into the water, which resulted in spectra that were not truly representative of the concentration of EDTA. EDTA has a pH of 4-6; to combat the issue of the EDTA dissolving, the pH was increased with 0.1 NaOH, allowing the EDTA to dissolve more easily. Both the 30 mg/ mL and 10 mg/mL EDTA standards showed absorption bands at approximately 1619 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1}, and 1322 cm^{-1}, all of which are consistent with the structure of EDTA (see Figures 5 and 6). These four absorption bands were expected throughout all of the EDTA concentrations that contained the structural peaks of interest. When the 6 mg/mL EDTA standard was analyzed however, the 1357 cm^{-1} and 1322 cm^{-1} peaks were observed but not called, likely due to the loss of intensity of the absorbance values. The threshold was set to 1.0 and the sensitivity was set to 50, so that some of the noise peaks were not called. These settings paired with the loss in absorbance intensity resulted in the 1619 cm^{-1} and 1401 cm^{-1} peaks being the only two peaks observed through all EDTA standard solutions. The same thing is observed with the spectrum from the 3 mg/mL standard. The 1.5 mg/mL EDTA standard, as seen in Figure 11, displayed the EDTA peaks of interest at 1619 cm^{-1} and 1401 cm^{-1}. As a result of the loss of intensity, the EDTA standards were prepared again, but with the 0.1 M liquid EDTA stock solution instead.

When compared to the spectra of the standards produced using the solid disodium EDTA, the standards prepared from the liquid EDTA solution yielded less background noise and a stronger intensity in the absorbance values for the peaks of interest, as seen in Figures 7-9. The peaks of interest are located at approximately 1619 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1}, and 1322 cm^{-1}. The peaks of interested with the highest absorbance intensities at 1619 cm^{-1} and 1401 cm^{-1} were observed in all but the 1.0 mg/mL standard (see Figures 7-11). Any standard solution with a

concentration of 1.0 mg/mL of EDTA or less did not have any peaks of interest, as shown in the 1.0 mg/mL FTIR spectrum (Figure 12).

For all of the data previously mentioned, the default FTIR parameters were used. The default FTIR parameters include 32 scans, 4 cm^{-1} resolution, an aperture of 100, and a mirror scan (optical velocity) value of 0.4747. In an attempt to increase sensitivity, the samples were all run using 64 scans (see Figures 7-12). There was an increase in sensitivity and subsequently in the intensity of the peaks of interest. Recording twice as many scans, does not double the signal to noise ratio. This is why 128 scans were not used for the spectra recorded regarding the EDTA standards. As a result, this setting was maintained throughout the rest of the experiment.

The optical velocity, or mirror speed, is the speed of the mirror that moves within the interferometer. The slower the velocity, the longer it takes for a sample spectra to be collected, but the result is a spectrum with higher intensities. When the mirror speed is increased, the resulting spectra has lower absorbances. The mirror speed was reduced to see if the intensity of the spectra would increase, making the EDTA absorption bands present at 1614 cm^{-1} and 1401 cm^{-1} more intense. The optical velocity was reduced from 0.4747 to 0.3165. The result was a slight increase in intensity of the two bands of interest. Figure 13 shows the 1.5 mg/mL EDTA standard sampled on the FTIR, but with a reduced optical velocity of 0.3165. There was an increase in the peaks of interest. However, there was much more noise present in the fingerprint region. Increasing the scans from 32 to 64 scans did increase the sensitivity, but it did so to both the peaks of interest at 1614 cm^{-1} and 1401 cm^{-1}, and the noise.

The resolution determines the minimum peak interval set that can be distinguished from one another. Therefore, the lower the resolution, the sharper the spectra will be. Resolution was another parameter that was altered, to identify whether it affected the intensity of the peaks of

interest. Figure 14 shows the difference between 32 and 64, with one having a resolution of 4 cm^{-1}, and the other having a resolution of 8 cm^{-1}. Noise is a random process; adding together an increasing number of scans will lead to a reduction in noise, because some of the noise will be cancelled out. Lowering the resolution, which lowers the minimum peak interval where a scan is taken of the sample, results in more scans being taken for the entire sample run. The lower the resolution, the higher the intensity of the peaks of interest, but the noise will likely increase as well. For the highest peak intensities without the noise being too high, the 4 cm^{-1} was used. Higher resolutions greatly reduced the intensities of the peaks of interest. The resolution was increased to 8 cm^{-1} with a reduced optical velocity, as seen in Figure 15. The result was no peaks of interest, only noise.

The aperture is an additional parameter that can be altered to adjust the spectra so that the absorption bands of interest can be isolated. The aperture determines how much infrared light hits the sample, and is set in relation to the resolution. The larger the aperture value, the higher an absorbance output will be. However, more noise will be present in the spectra as well. The aperture was reduced to 87 instead of the default 100 to try to reduce the noise present in the EDTA standards at concentrations of 3 mg/mL or lower. Because the aperture and resolution are set in relation to each other, decreasing the aperture meant that the resolution could be set to 2 cm^{-1} instead of the default 4 cm^{-1}. Decreasing the aperture removed the absorption bands of interest, while also removing some of the noise (see Figures 16-18). Therefore, it was determined that the default aperture of 100 should be used when confirming the presence of EDTA.

The final FTIR parameters determined from these experiments were as follows: 64 collected scans, 4 cm^{-1} resolution, aperture of 100, and the optical velocity of 0.4747. Figure 19 displays five replicates of the 30 mg/mL EDTA solution prepared from the liquid EDTA stock,

which is much cleaner, with less noise present in the 2200-2000 cm^{-1} range. The 30 mg/mL EDTA standard solution has all four bands of interest, located at 1619 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1}, and 1323 cm^{-1}. The common scale was what was used for all resulting spectra, because it normalized the peaks of interest better than full scale, which normalized to the tallest peak (see Figure 19).

The EDTA standard concentrations of 30 mg/mL and 10 mg/mL produced all four peaks of interest, as seen in Figures 19 and 20. The EDTA concentrations of 3 mg/mL and 1.5 mg/mL only had the two peaks of interest, at approximately 1615 cm^{-1} and 1401 cm^{-1} (Figures 21-22). The 1.0 mg/mL EDTA standard did not produce peaks of high enough intensities to be called. Therefore, the detection limit of EDTA in water using FTIR is approximately 1.5 mg/mL. This is much higher than expected, because lower concentrations of EDTA can be detected using UV-Vis spectroscopy [4,5]. It is likely because of the water interference that is observed when using FTIR, in combination with the detection limit of the instruments used for this experiment.

A standard linear regression plot was performed on the most abundant peak in the FTIR spectra (i.e., the peak present at ~ 1615 cm^{-1}), for each of the concentrations that contained visible peaks associated with the EDTA. The purpose of the linear regression was to quantify the relationship between the absorbance and the concentration, that is, to identify whether the changes in the absorbance values linearly proportional to changes in the concentration of EDTA . The linear regression provides the Pearson's correlation coefficient and the R^2 value. Pearson's correlation coefficient, or r, identifies the correlation between two quantities. The R^2 value is used to associate these quantities to the linear regression line. It expresses how well the variance in the absorbance values is explained by the change in the concentration of the EDTA standards. The closer the value is to 1, the more the variance can be explained.

The linear regression had an R^2 value is 0.993, which expresses that 99.3% of the variance in the absorbance is explained by the increase in the concentration of the EDTA standards. The correlation coefficient of 0.997 indicates that there is a strong relationship between the absorbance and the concentration of the standards, as seen in Figure 23. For linear regressions, the null hypothesis is that there is no correlation between the values. That can be rejected for this experiment.

The signal to noise ratio for the two most abundant peaks present in the FTIR spectra (1615 cm^{-1} and 1401 cm^{-1}) was calculated for each of the standard solution concentrations where the EDTA was present. The signal to noise ratio is defined as the average of the peak signal over time, divided by the noise. The noise in an FTIR signal is determined by the root mean squared value of the output fluctuations that occur over time. For each of the peaks of interest, the average absorbance of the peak was calculated from the replicate data for each of the concentrations, which was then divided by the standard deviations of those values. The only signal to noise ratios that were less than 2.5 were those associated with the peaks in the 1.5 mg/mL EDTA standard, as seen in Table 1. Usually, a signal to noise ratio of at least 3 is ideal. Many of the signal to noise ratios are around 3 or higher. The 30 mg/mL EDTA standard had signal to nose ratios of at least 3, and the 10 mg/mL EDTA standard had signal to noise ratios of at least 8. The 3 mg/mL EDTA standard had signal to noise ratios of 3.94 and 3.40 for the 1615 cm^{-1} and 1401 cm^{-1} peaks, respectively.

Different substrates were briefly analyzed to see how they would affect the detection of the EDTA bands of interest. Cotton swabs are the most commonly used substrate to collect potential biological fluid from the crime scenes and/or evidentiary samples. Foam swabs are a suitable alternative to the cotton swabs, as well as filter paper. The idea was that if EDTA

detection was possible on the substrates, then there would be no sample preparation required. It would remove the preparation involved in re-constituting the sample into a liquid and would prevent further dilution of the evidence. Analyzing the dried standards and samples on the substrates would also remove the water interference commonly experienced in FTIR. In terms of the filter paper, the only preparation required would be pipetting the liquid blood sample onto the filter paper and letting it air dry for at least an hour. Filter paper is used for FTIR analysis of trace evidence like fire debris and explosive compounds.

Foam swabs containing 30 mg/mL or 10 mg/mL EDTA standard produced spectra consistent with a polypropylene product (see Figures 24-26). Initially, the blank of the foam swab was subtracted out as the background. The spectra collected appeared identical to that of the substrate, but with negative absorbance values (see Figure 26). The blank absorbed at values higher than the sample, which was why the sample spectra contained negative values. To try to combat this issue, air was collected as the background, as seen in figure 4, and subtracted from the sample spectra of both the foam swab and the EDTA. The EDTA concentration was too low, and resulted in bands with absorbances too low to be seen over the bands from the foam swab substrate. Only the absorption bands affiliated with the polypropylene swab were observable.

The filter paper and the cotton swabs were then tested as substrates, using the EDTA concentrations of 3-30 mg/mL. However, none of the EDTA peaks of interest at any standard concentration were observed. The absorption bands present at 1000 cm^{-1}, 2900 cm^{-1}, and 3300 cm^{-1} are indicative of this. The only peaks reported corresponded to the items being cellulose-based products (see Figures 27-31). Blood was pipetted onto the filter paper, but EDTA peaks were no observed(Figure 29). Detected bands represented the C-O bond, the C-H bond, and the O-H bond, respectively [20]. The bands present in the cotton blank were at absorbance values

similar to the filter paper (see Figure 31), so it was anticipated that the EDTA standards would continue to be undetectable. Since the EDTA in the standards, including the highest concentrations, were not detectable when deposited onto a substrate, no other substrates were tested during this experiment.

The 1.5 mg/mL EDTA in blood samples were analyzed in liquid form as the last part of the FTIR method of this experiment. The EDTA peaks could not be isolated when the EDTA was mixed with a blood sample. The amide I and II peaks are in much higher concentrations than the concentration of the EDTA itself. Both water and whole blood without EDTA were subtracted out as the background, to see if the absorbance values of the amide I and II peaks would change significantly. The EDTA peaks of interest continued to be undetectable. Blood dilutions were used, to see if the 1.5 mg/mL EDTA in blood samples were not suitable for the identification of the EDTA, as seen in Figures 32-35. Although not forensically relevant, a blood sample was spiked with EDTA at a concentration of 30 mg/mL, to try to see if the very high EDTA concentration would allow the peaks to be observable, without removing the amide I and II peaks in the blood. The result was that the spectra looked exactly like the whole blood with no EDTA and whole blood with 1.5 mg/mL EDTA.

Raman

The method discussed for the identification of EDTA using Raman spectroscopy was not suitable in detecting EDTA in both the EDTA standards and blood samples. The interference from the glass slide overpowered any of the EDTA peaks of interest. This was an issue for both the standards and the blood samples. To try to reduce the interference of the glass slide, the laser power and aperture were reduced to below the 10 mW and 25 μm sizes. All changes to the Raman parameters were unsuccessful, with the EDTA being undetectable. The same analysis

was conducted on a 1.5 mg/mL EDTA in blood sample, and there was no success. All of the resulting spectra were indistinguishable from the glass substrate blank (Figure 36).

Conclusion

Individuals may plant blood evidence at a crime scene to implicate another person. Unless fresh blood is collected and planted at the scene, the blood evidence that is planted may have an anticoagulant in it, so that the blood does not clot. There is a need for a confirmatory method to identify EDTA in blood samples, especially one that requires little to no preparation, and does not involve further diluting a blood sample. This study demonstrates that EDTA standards ranging from 1.5-30 mg/mL diluted in water can be identified using FTIR spectroscopy. The low end of this detection range is similar to that of normal concentrations of EDTA in collected blood samples (1-1.5 mg/mL). Because forensically relevant samples containing EDTA will likely be diluted to concentrations lower than 1.0 mg/mL, the current method developed is not suitable for samples expected at crime scenes. The established methods were not suitable for isolating the EDTA absorption bands from the amide I and II absorption bands associated with blood. Therefore, without additional optimization, EDTA cannot be identified in blood samples at forensically relevant concentration ranges. Raman spectroscopy is not suitable at this time for the identification of EDTA in both standards diluted in water and blood samples that were diluted with either additional blood or water.

The results from this experiment identified that FTIR and Raman spectroscopy may not be suitable instrumental methods for the confirmation of EDTA in blood samples. This benefits the field of forensic science, because it has established that different, more sensitive confirmatory methods like HPLC-MS may be more suitable. HPLC-MS is destructive and

requires more sample preparation, but it has more capabilities of confirming the presence of EDTA in samples with concentrations lower than expected in pre-prepared EDTA tubes (1-1.5 mg/mL).

Future directions of this research include troubleshooting and optimizing the Raman spectroscopic method, specifically determining whether the EDTA in blood can be identified. The tubes used to hold liquid samples may be a more suitable substrate than the glass microscope slides. With regards to the FTIR spectroscopic method, the future directions include seeing if the sensitivity to EDTA can be improved, and if the peaks of interest associated with the EDTA can be isolated from the amide I and II peaks in the blood samples. However, the expected detection limit of EDTA using both FTIR and Raman spectroscopy is approximately 1.0 mg/mL. It is likely that these methods will not be suitable for forensically relevant EDTA samples. HPLC-MS and GC-MS are more sensitive methods, although they are destructive and require time consuming sample preparation. In addition, there are other anticoagulants commonly used, like potassium EDTA, sodium citrate, and heparin. Both Raman and FTIR could be used to analyze other anticoagulants, since it's possible that the amide peaks in blood will not interfere with the spectra of different anticoagulants. A final additional study would be to examine samples from multiple donors and see if there are any factors that impact the identification of EDTA in a blood sample.

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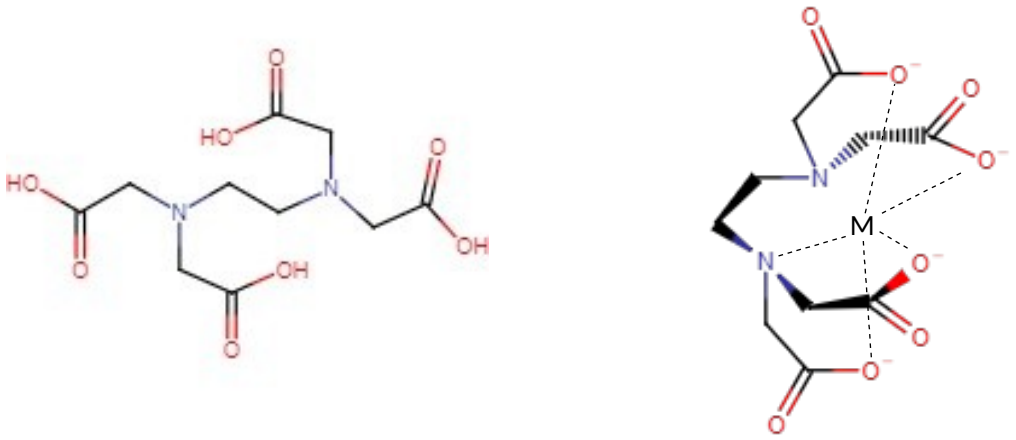
Figures and Tables:



a) **b)**

Figure 1: Structure of EDTA

EDTA is displayed in both its (a) unbound and (b) bound structure. When bound, EDTA chelates a single metal ion, which could be magnesium or calcium in the context of blood.[4,5]

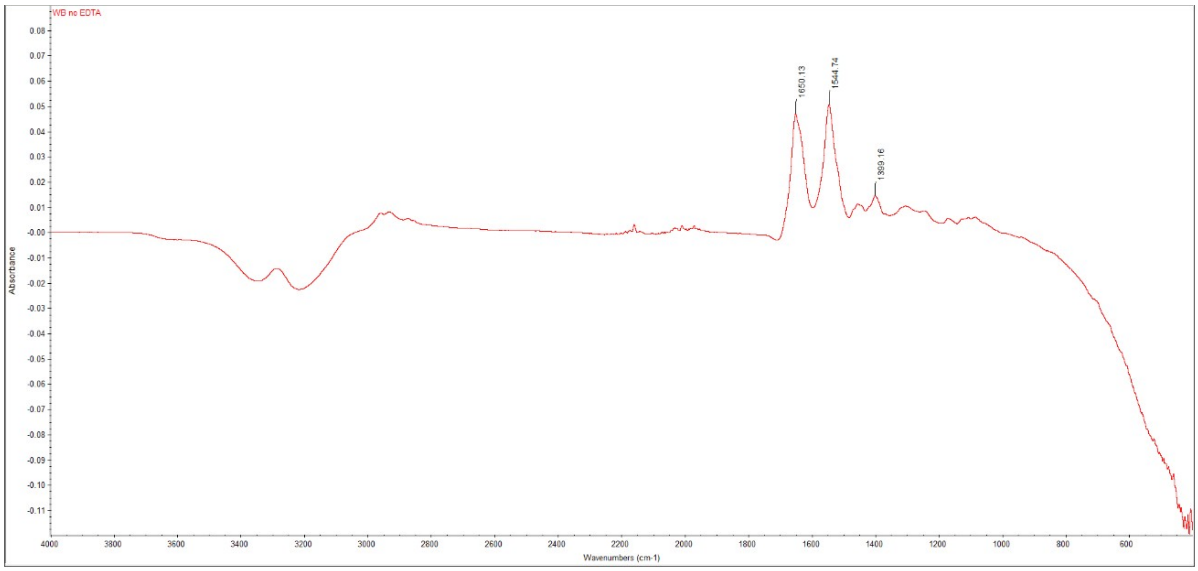


Figure 2: FTIR Spectrum of Whole Blood with no EDTA

Above is a FTIR spectrum of whole blood with no EDTA present. The absorpion bands of interest are the amide I and II bands reported at 1650 cm^{-1} and 1544 cm^{-1}.

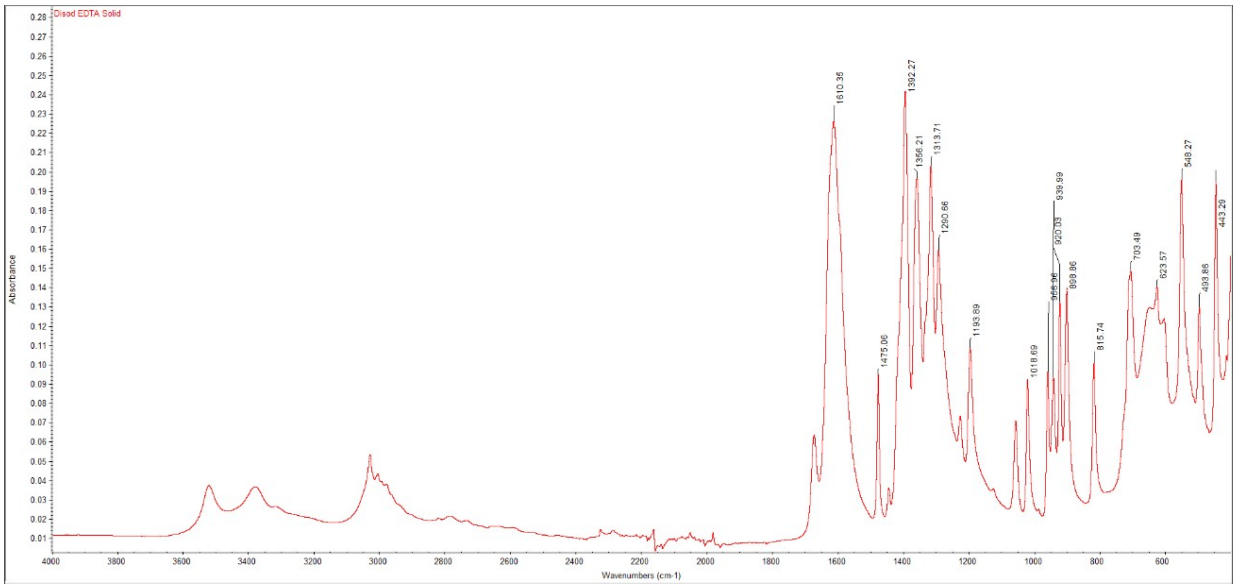


Figure 3: FTIR spectrum of solid disodium EDTA

Above *is the FTIR spectrum of a solid disodium EDTA sample. The bands of interest are at 1610 cm^{-1}, 1392 cm^{-1}, and 1356 cm^{-1}. The spectra was collected using 32 scans.*

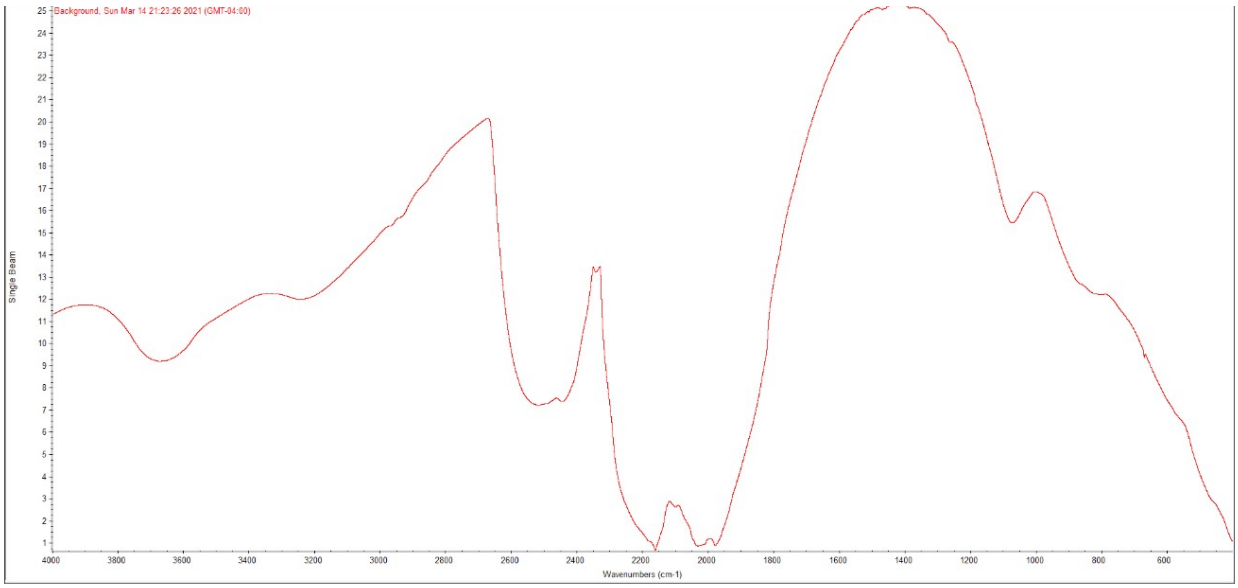


Figure 4: FTIR Spectrum of Air Background

Above is the air background that was subtracted out of various sample spectra, specifically for the solid EDTA sample and some of the samples run for the substrate study.

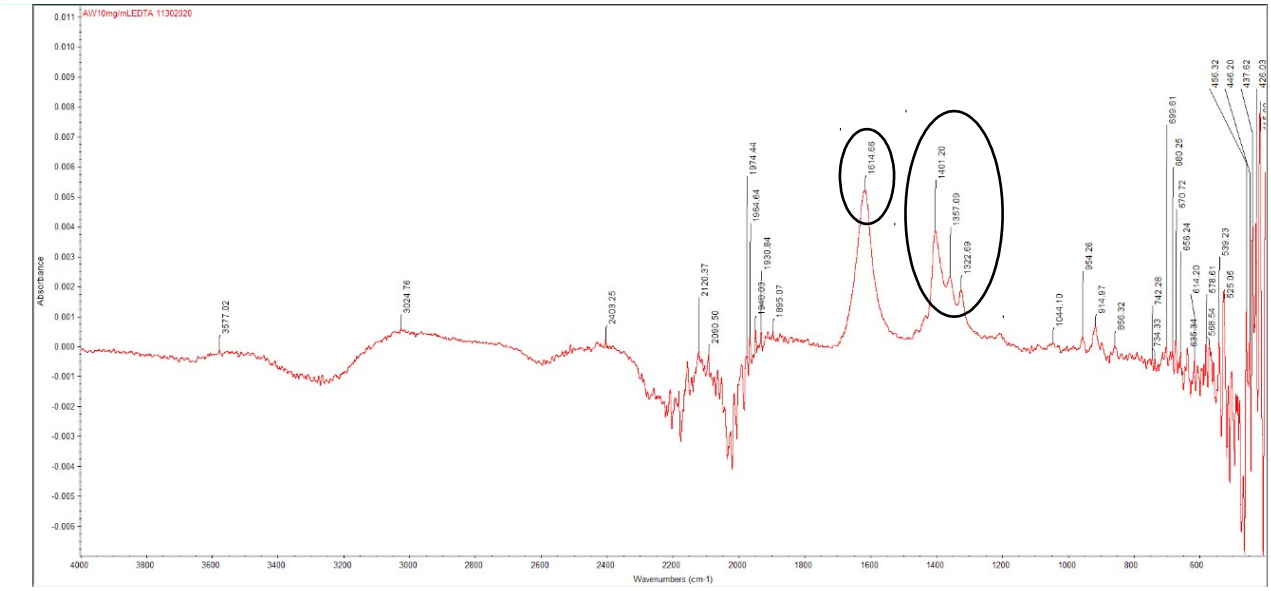


Figure 5: FTIR Spectrum of 10 mg/mL EDTA standard in water using solid EDTA

Above is a FTIR spectrum of the 10 mg/mL EDTA standard that was prepared using HPLC grade water and solid disodium EDTA. The bands of interest are at 1614 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1}, and 1322 cm^{-1}. Overall, 32 scans were collected.

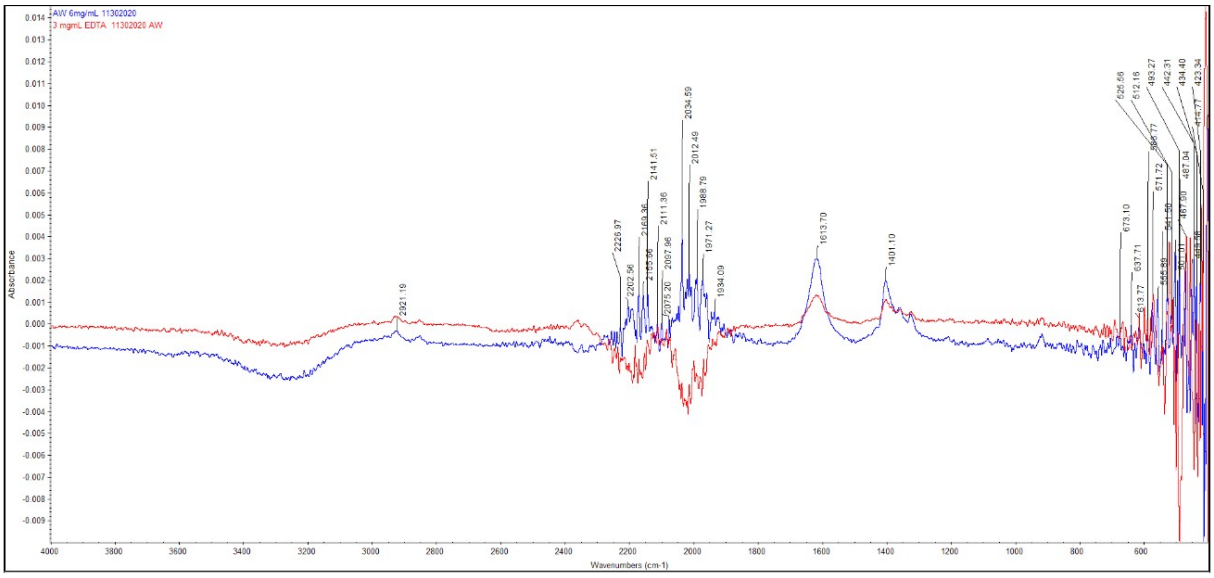


Figure 6: FTIR Spectra 6 mg/mL and 3 mg/mL EDTA standards using solid EDTA

Above is a superimposed FTIR spectra of the 6 mg/mL (blue) and 3 mg/mL (red) EDTA standards that were prepared using solid disodium EDTA. The bands of interest are reported at 1613 cm^{-1} and 1401 cm^{-1}. In total, 32 scans were collected.

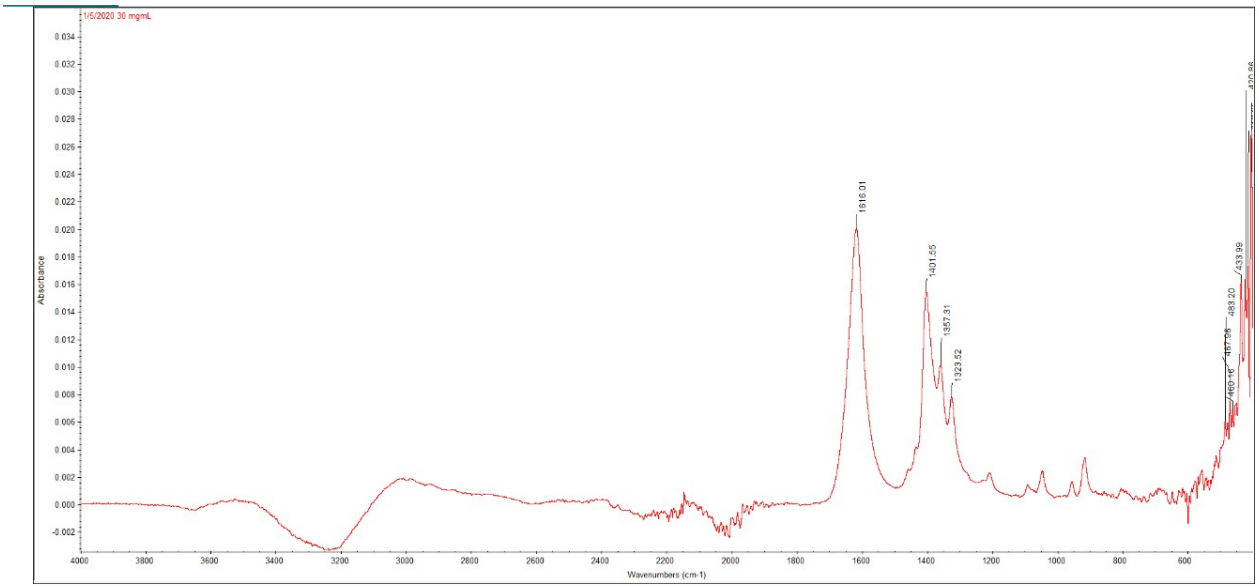


Figure 7: FTIR spectrum of 30 mg/mL EDTA standard

Above is a spectrum of the 30 mg/mL EDTA standard using a purchased liquid EDTA standard solution. Collected scans were increased to 64, which increased the sensitivity and the intensity of the EDTA absorption bands. The bands of interest are reported at 1616 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1}, and 1323 cm^{-1}.

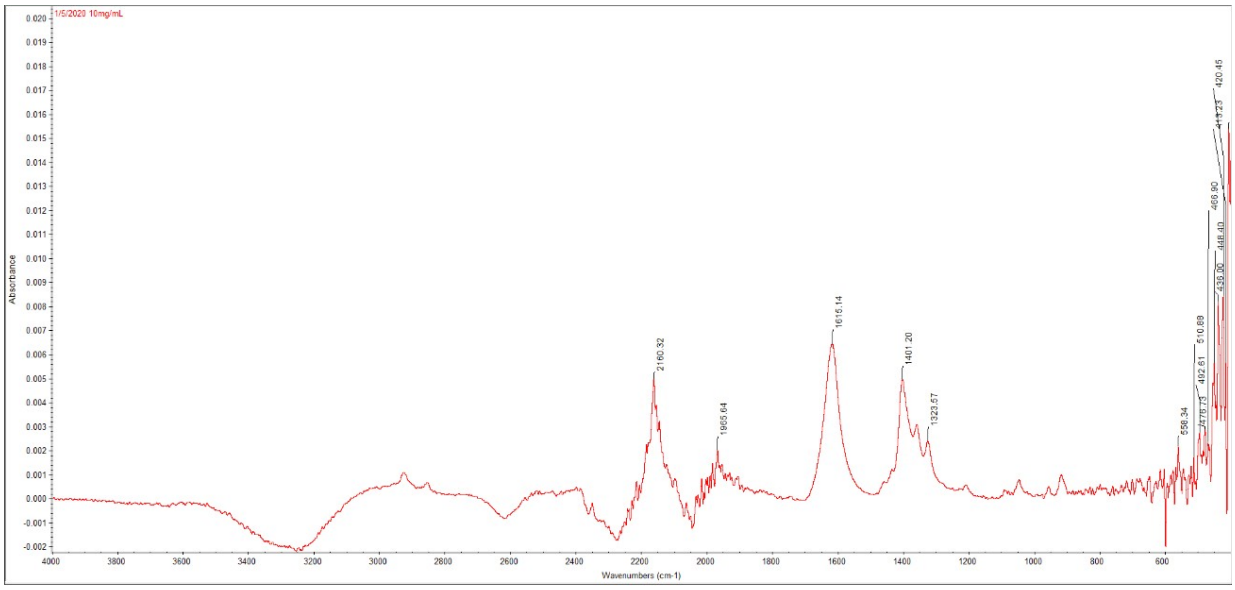


Figure 8: FTIR spectrum of 10mg/mL EDTA standard

Above is the FTIR spectrum of the 10 mg/mL EDTA standard that was prepared using the liquid EDTA standard solution. The bands of interest are at 1615 cm^{-1}, 1401 cm^{-1}, and 1323 cm^{-1}. In total, 64 scans were collected.

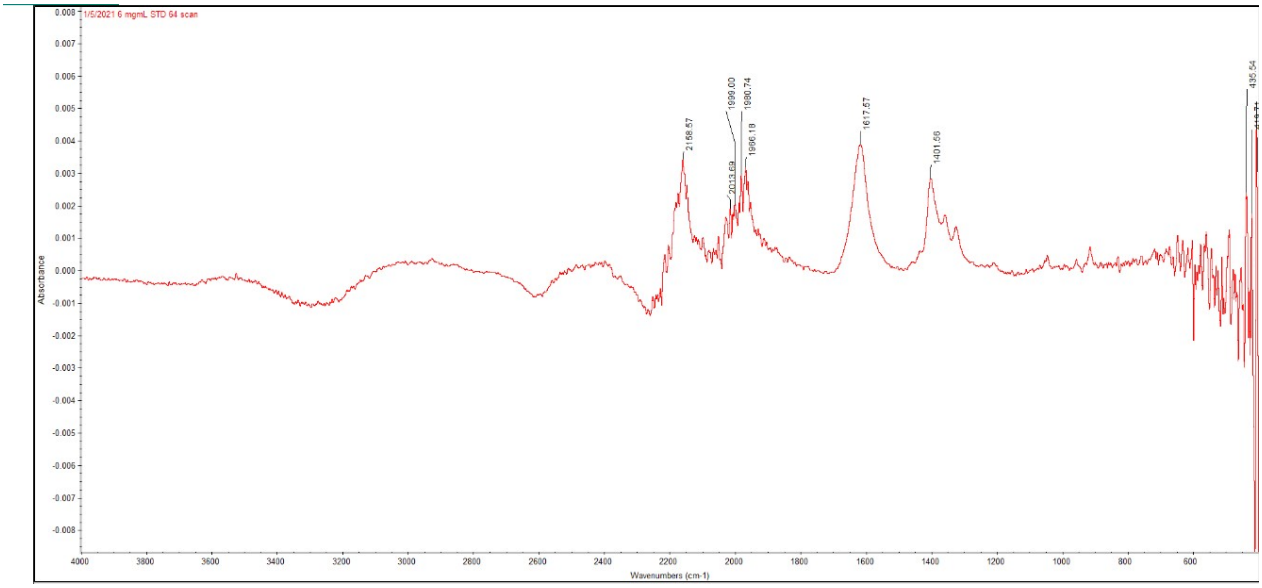


Figure 9: FTIR spectrum of 6 mg/mL EDTA standard

Above is the FTIR spectrum of the 6 mg/mL EDTA standard that was prepared using the liquid EDTA standard solution. The bands of interest are at 1615 cm^{-1} and 1401 cm^{-1}. In total, 64 scans were collected.

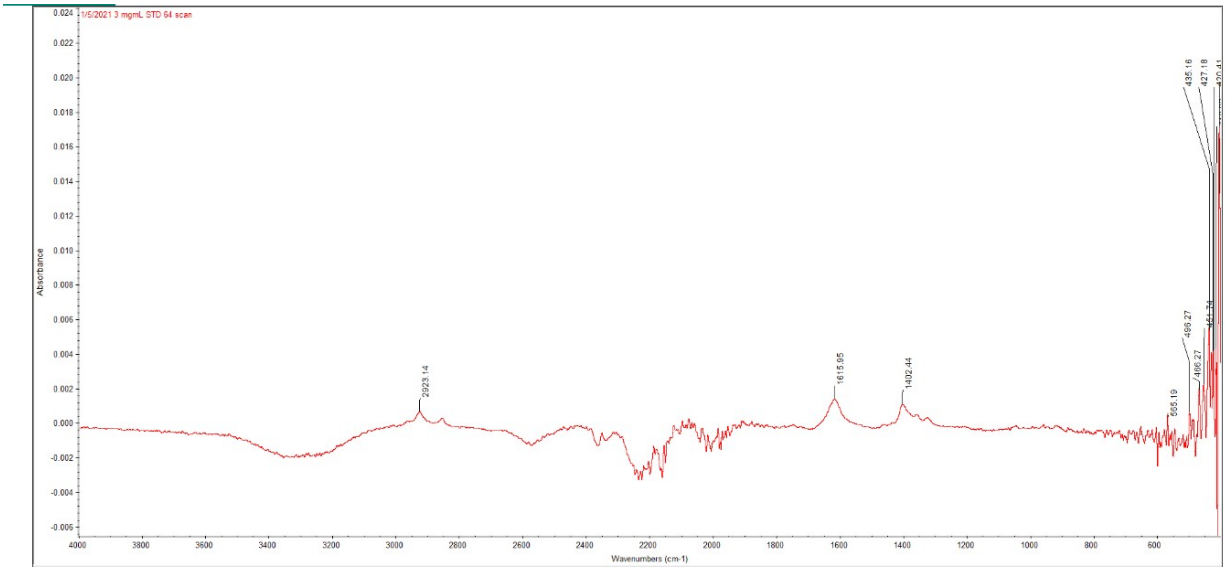


Figure 10: FTIR spectrum of 3 mg/mL EDTA standard

Above is the FTIR spectrum of the 3 mg/mL EDTA standard that was prepared using the liquid EDTA standard solution. The bands of interest are at 1615 $cm^{-1}$and 1402 cm^{-1}. In total, 64 scans were collected.

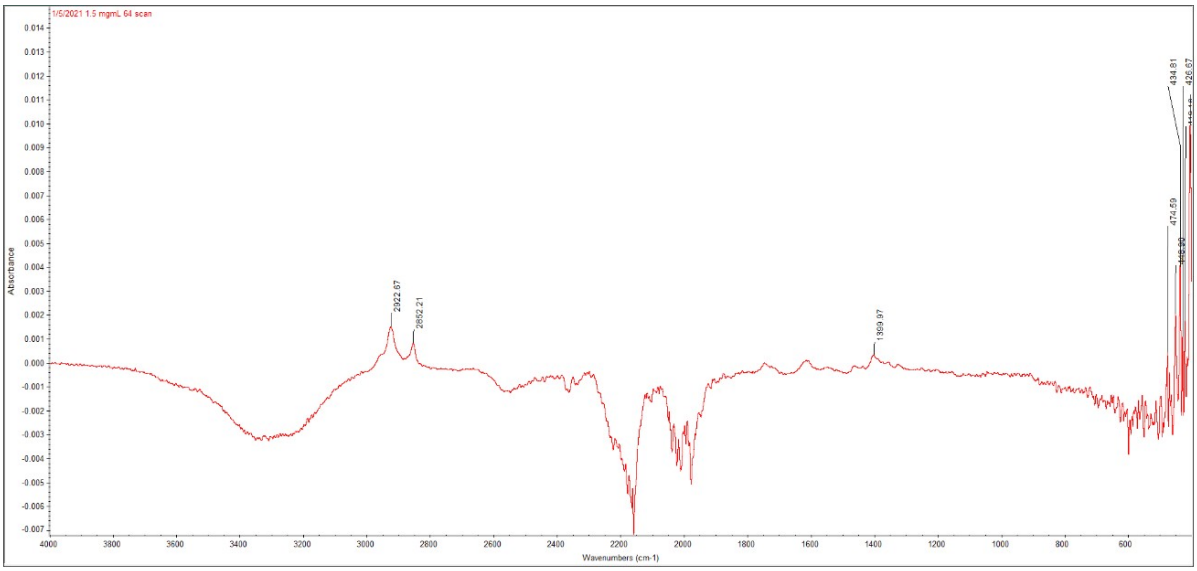


Figure 11: FTIR spectrum of 1.5 mg/mL EDTA standard

Above is the FTIR spectrum of the 1.5 mg/mL EDTA standard that was prepared using the liquid EDTA standard solution. The peak of interest is at 1399 cm^{-1}. Overall, 64 scans were collected.

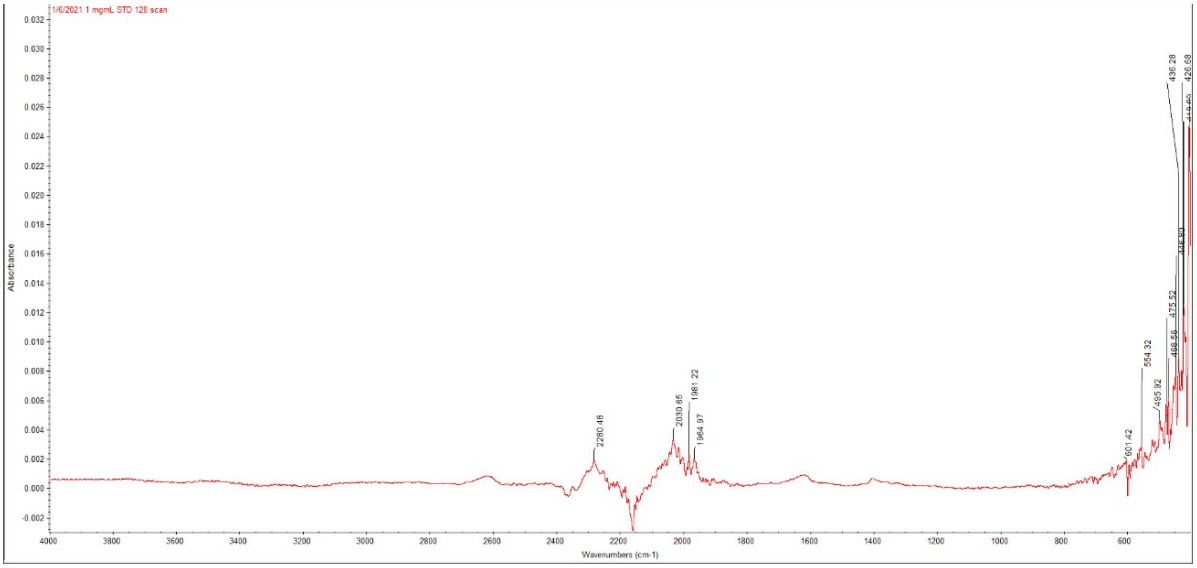


Figure 12: FTIR spectrum of 1.0 mg/mL EDTA standard

Above is the FTIR spectrum of the 1.0 mg/mL EDTA standard that was prepared using the liquid EDTA standard solution. The bands of interest were not detected. In total, 128 scans were collected.

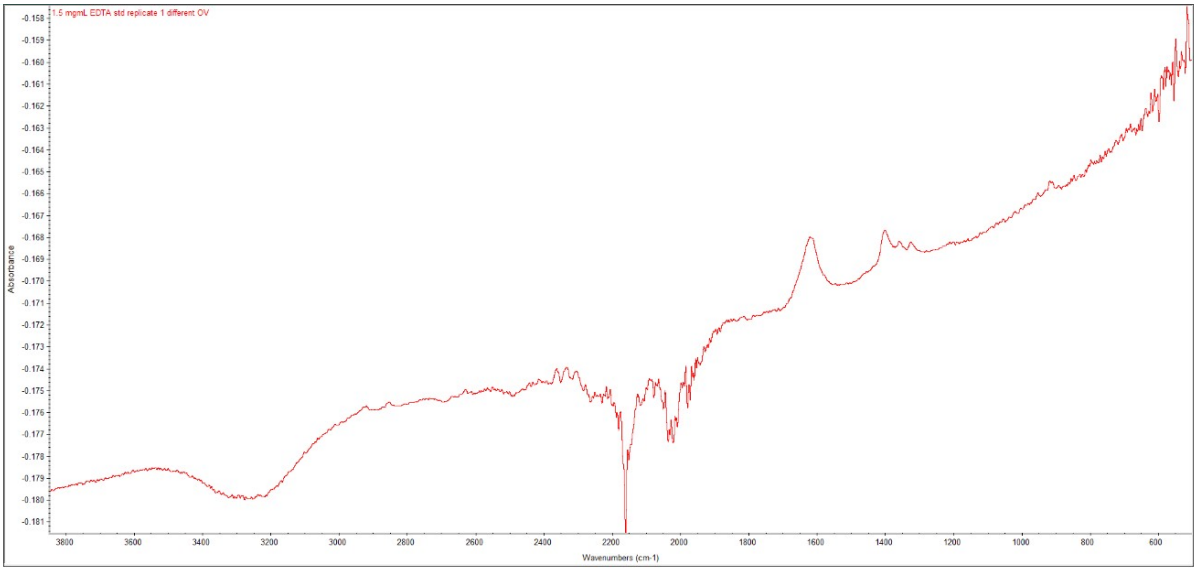


Figure 13: FTIR spectrum of 1.5 mg/mL EDTA standard collected with a reduced optical velocity

Above is a FTIR spectrum of the 1.5 mg/mL liquid EDTA standard; it was collected using a reduced optical velocity of 0.3165, versus the standard 0.4747 optical velocity. In total, 64 scans were collected. The peaks are present, but the noise is too high for the peaks to be called.

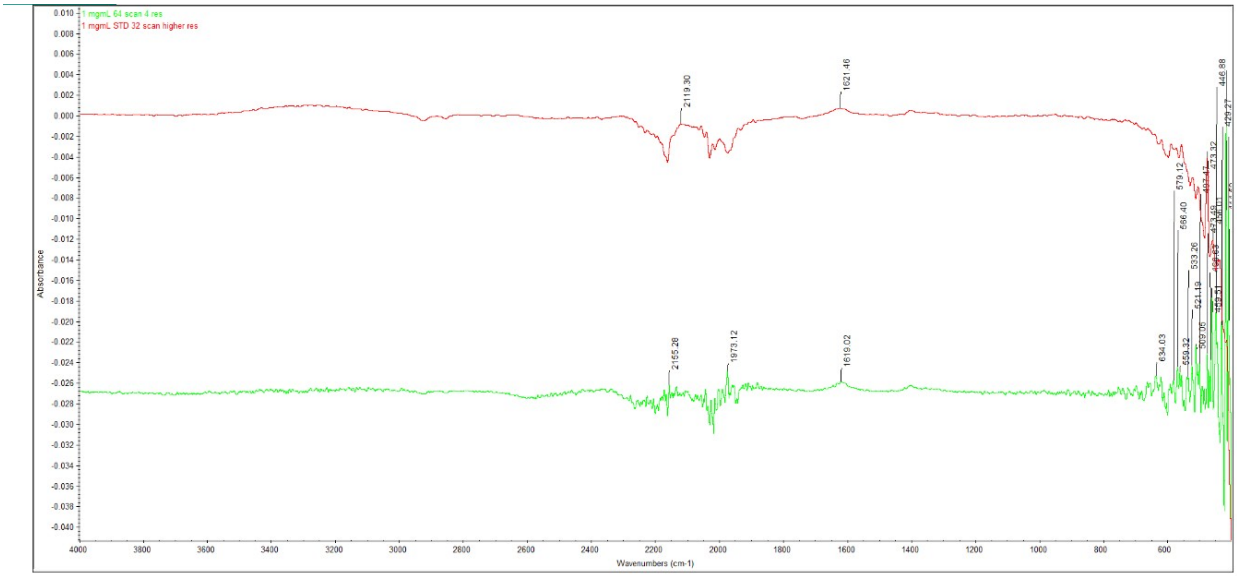


Figure 14: FTIR spectra of 1.0 mg/mL EDTA standard

Above is the FTIR spectra of the 1.0 mg/mL EDTA standard with 32 scans collected, overlayed with the 1.0 mg/mL standard collected using 64 scans and an 8 cm^{-1} resolution. Band of interest is at 1619 cm^{-1}. The peak is at intensities lower than the noise, which indicates that this may be below the detection limit of the instrument.

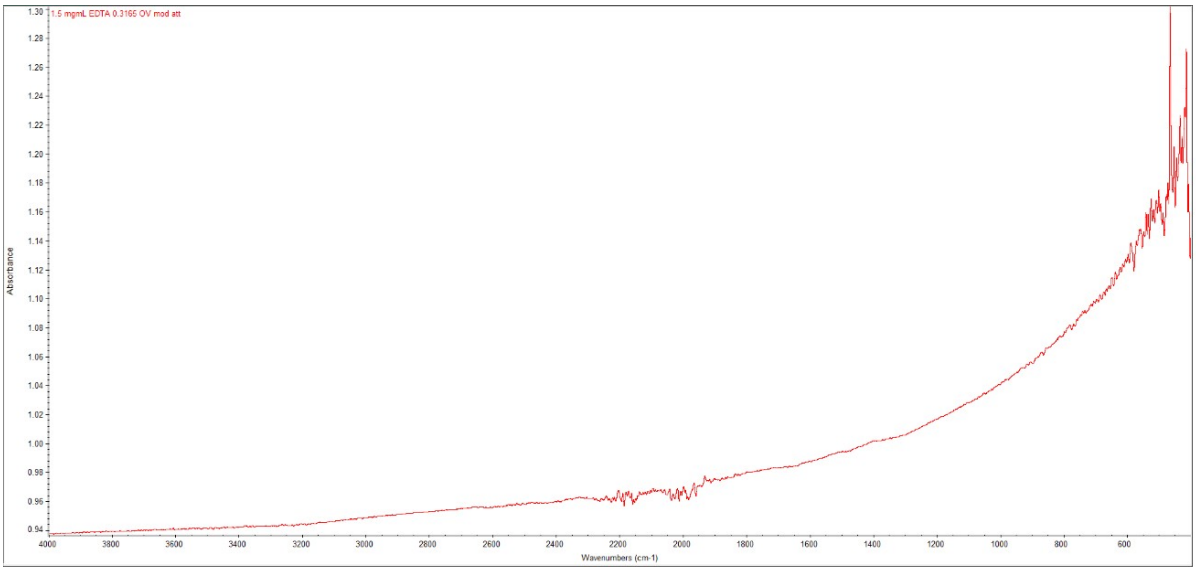


Figure 15: FTIR spectrum of 1.5 mg/mL EDTA standard collected with reduced optical velocity and different attenuation settings

Above is a FTIR spectrum of the 1.5 mg/mL EDTA standard; it was collected using 8 cm^{-1} resolution, moderate attenuation, a reduced optical velocity of 0.3165, and a collection of 64 scans; no peaks of interest of observed.

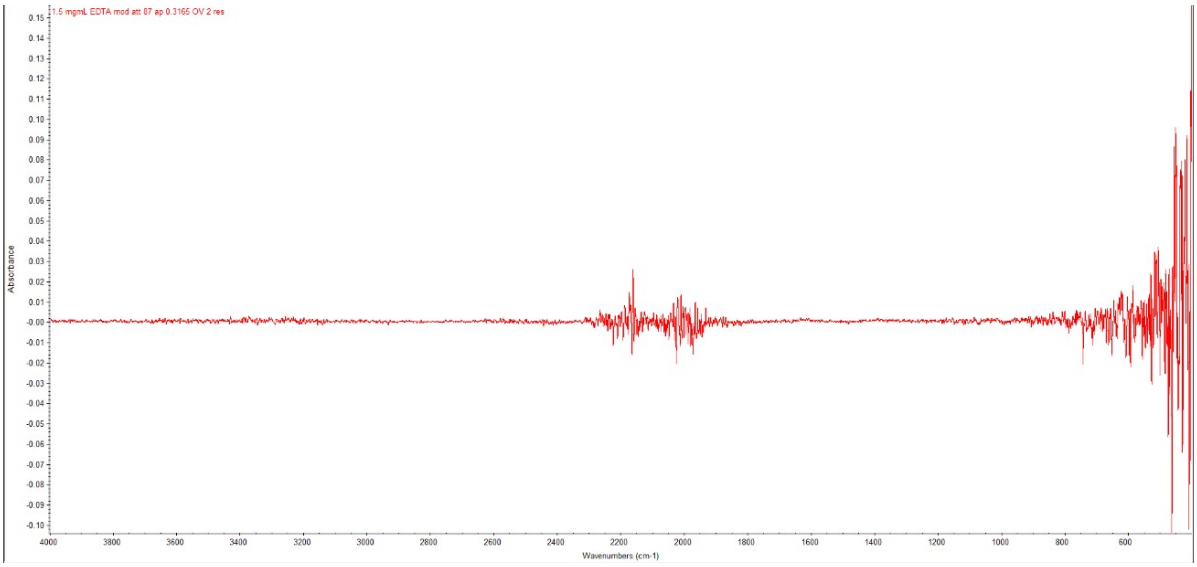


Figure 16: FTIR Spectrum of 1.5 mg/mL EDTA standard collected using a 2 cm^{-1} resolution, reduced aperture, moderate attenuation, and reduced optical velocity

Above is a FTIR spectrum of the 1.5 mg/mL EDTA standard. It was collected using various altered FTIR parameters including a 2 cm^{-1} resolution, a reduced aperture to 87, moderate attenuation, a 0.3165 optical velocity, and a collection of 64 scans. There are no absorption bands of interest, as the sensitivity to the EDTA peaks of interest was not increased.

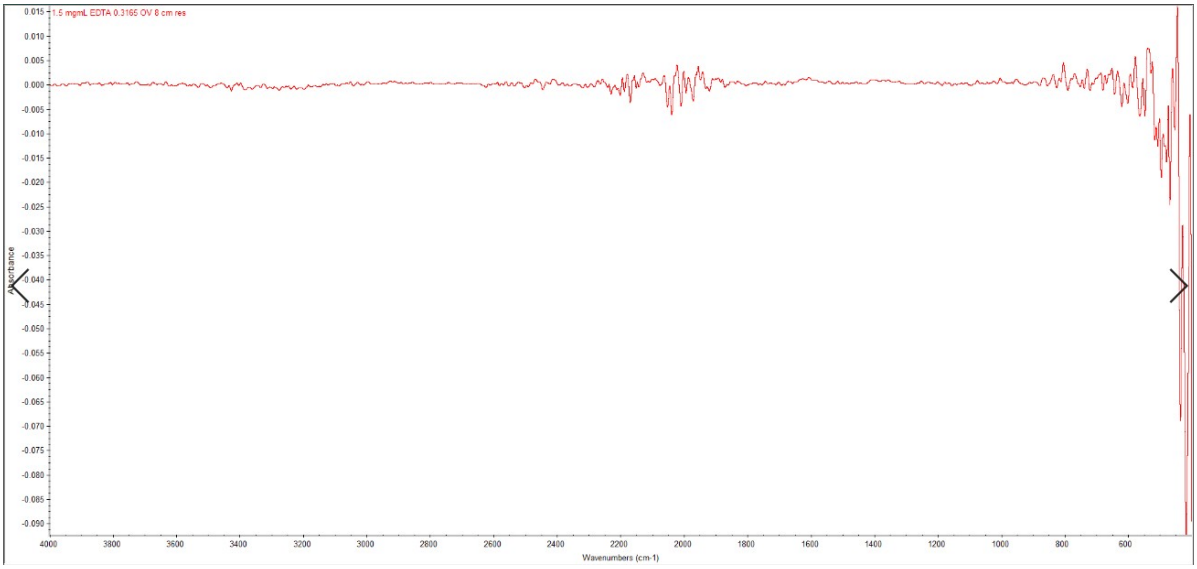


Figure 17: FTIR spectrum of 1.5 mg/mL EDTA standard collected using 8 cm^{-1} resolution, reduced optical velocity and moderate attenuation

Above is a FTIR spectrum of the 1.5 mg/mL EDTA standard. It was collected using an optical velocity of 0.3165, moderate attenuation, and an 8 cm^{-1} resolution. In total, 64 scans were collected. No peaks of interest are observed; the proposed method did not increase the intensity of the EDTA peaks of interest.

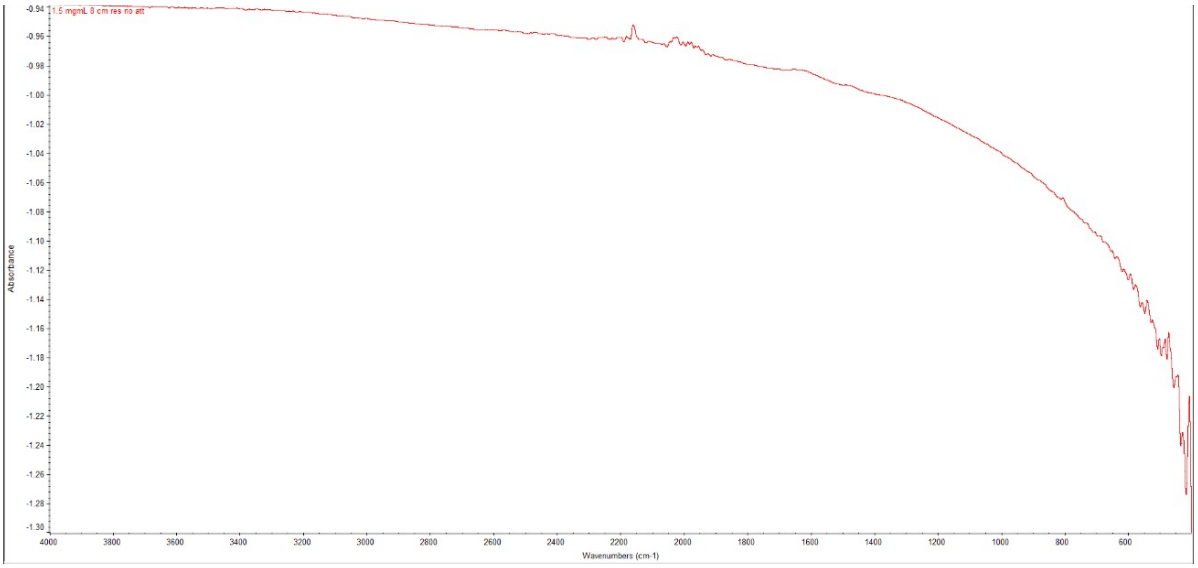
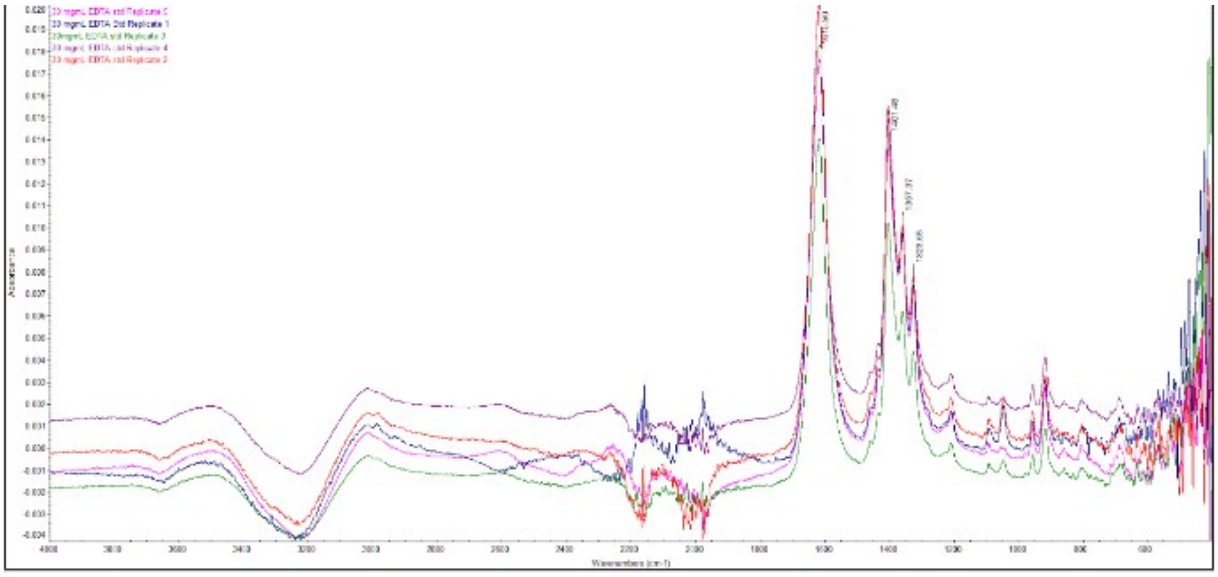
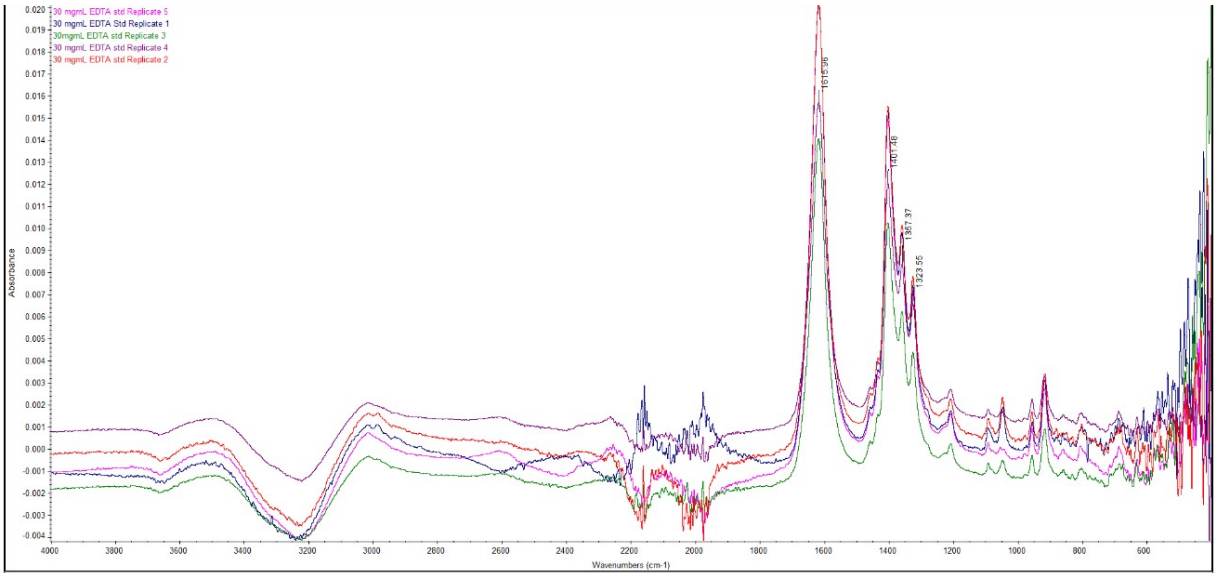


Figure 18: FTIR spectrum of 1.5 mg/mL EDTA with 8 cm^{-1} resolution and no attenuation

Above is an FTIR spectrum of the 1.5 mg/mL EDTA standard. It was collected using an 8 cm^{-1} resolution, and the instrument was set to no attenuation. In total, 64 scans were collected. There are no absorption bands of interest present.



a)



b)

Figure 19: FTIR spectrum of five replicates of 30 mg/mL liquid EDTA

Above is an FTIR spectrum of five replicates of the 30 mg/mL EDTA standard corrected to full scale, (a), and common scale (b). For all, 64 scans were recorded with a 4 cm^{-1} resolution. The

absorption bands of interest are at 1615 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1} and 1323 cm^{-1}. When common scale was used, the peaks were tighter and more normalized to each other.

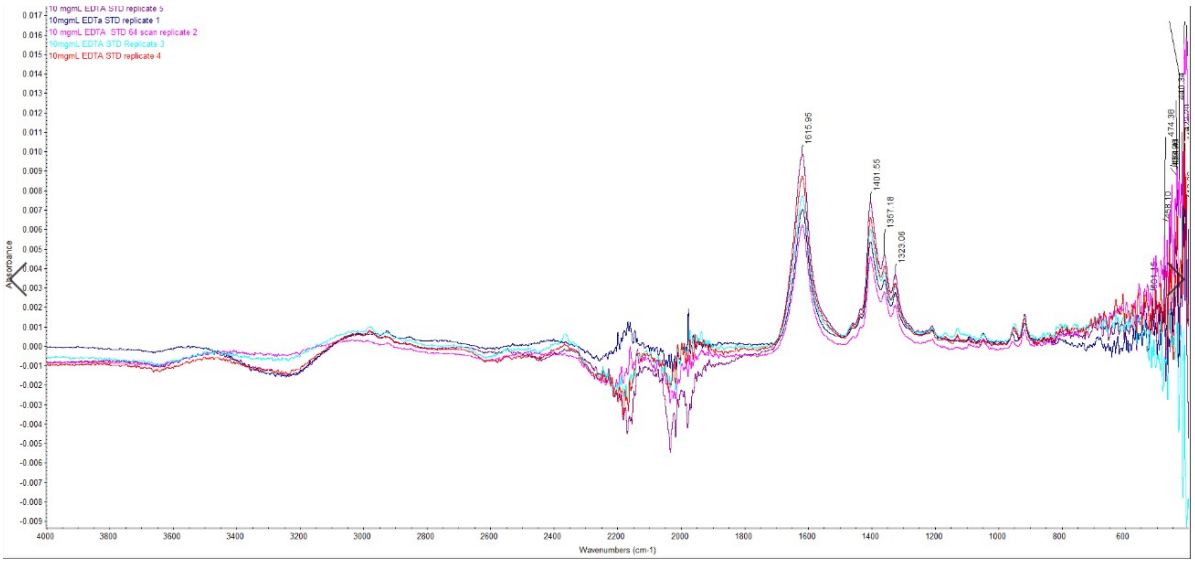


Figure 20: FTIR spectra of five replicates of 10 mg/mL EDTA standard

Above is an FTIR spectrum of the 10 mg/mL EDTA standard corrected to common scale. A total of 64 scans were recorded with a 4 cm^{-1} resolution. The bands of interest are at 1615 cm^{-1}, 1401 cm^{-1}, 1357 cm^{-1} and 1323 cm^{-1}.

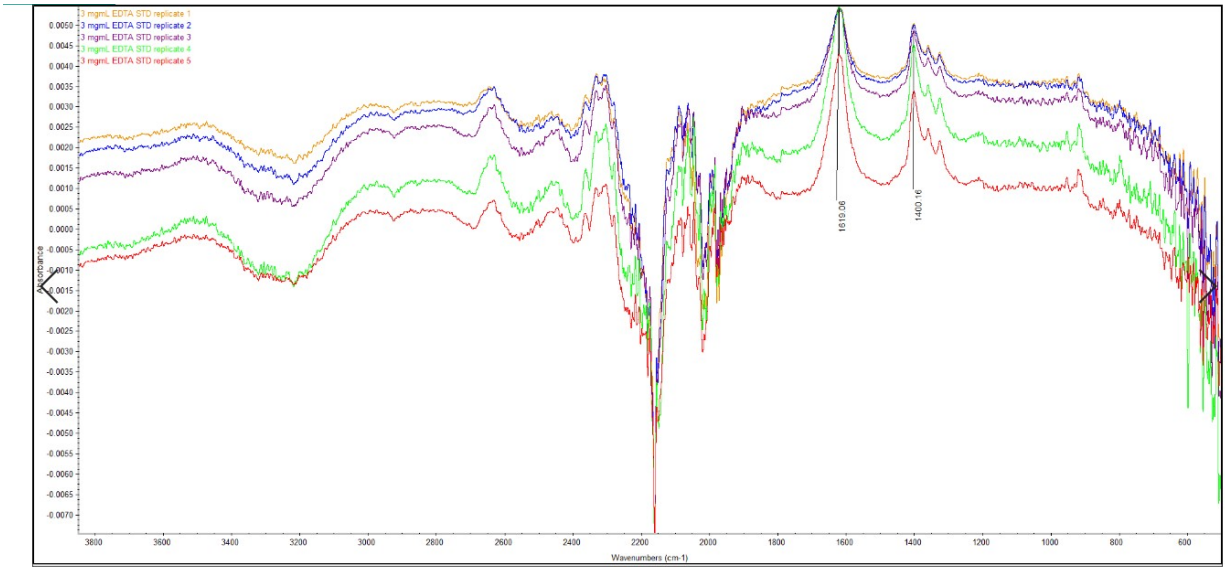


Figure 21: FTIR spectra of five replicates of 3 mg/mL liquid EDTA standard

Above is an FTIR spectrum of five replicates the 3 mg/mL EDTA standard corrected to common scale. In total, 64 scans were recorded with a 4 cm^{-1} resolution. The bands of interest are at 1615 cm^{-1} and 1401 cm^{-1}.

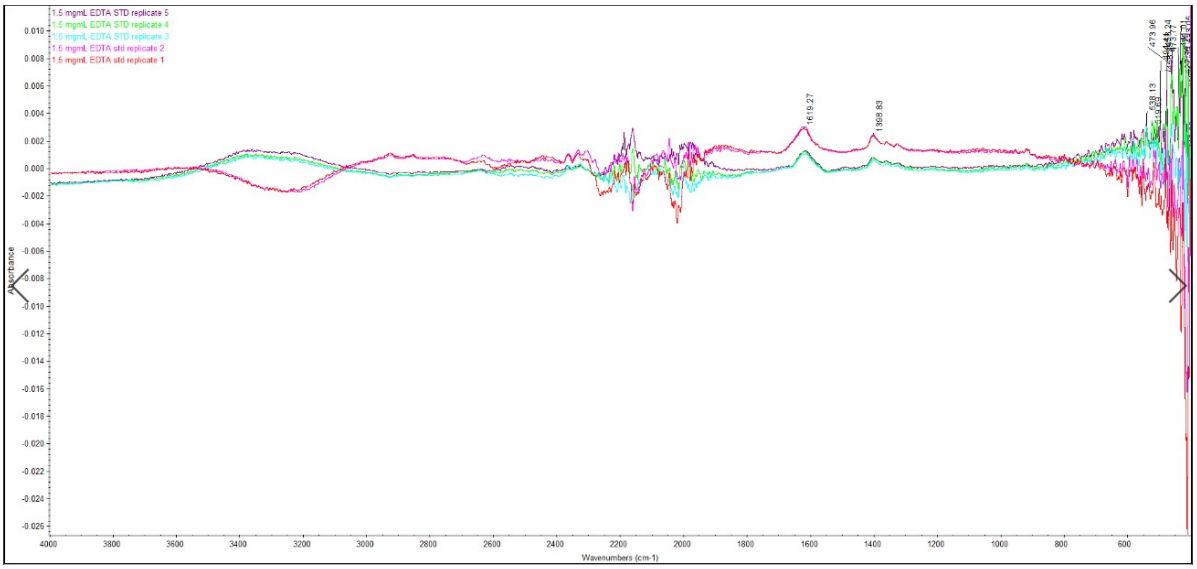


Figure 22: FTIR spectra of five replicates of 1.5 mg/mL liquid EDTA standard

Above is an FTIR spectrum of the 1.5 mg/mL EDTA standard corrected to common scale. A total of 64 scans were recorded with a 4 cm^{-1} resolution. The bands of interest are at 1615 cm^{-1} and 1401 cm^{-1}.

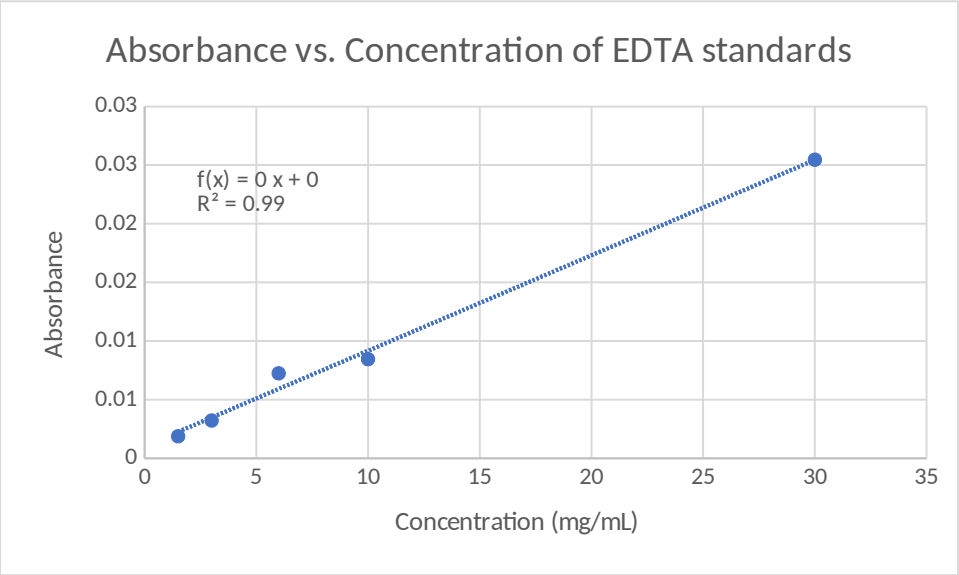


Figure 23: Linear Regression of Absorbance Values vs. Concentration of EDTA standards

Above is a linear regression of the absorbance values versus the concentration of the EDTA standards that produced an EDTA peak at 1615 cm^{-1}. There was a positive correlation between the two variables: $r = 0.997$, and $R^2 = 0.993$.

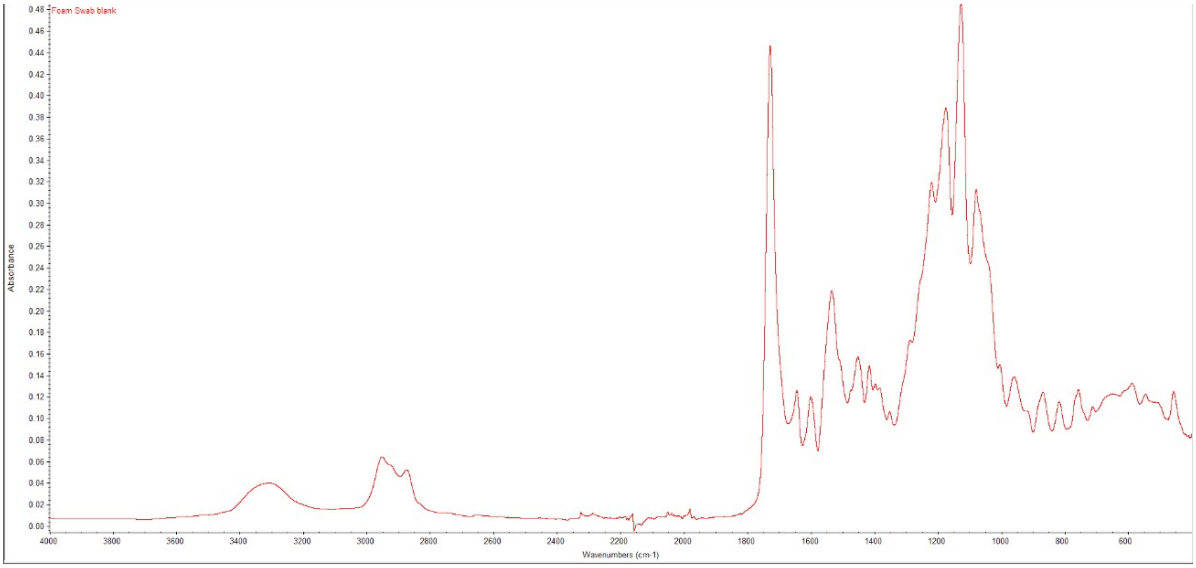


Figure 24: FTIR spectrum of Foam Swab Blank

Above is the FTIR spectrum of the foam swab substrate. The foam swab is consistent with a polypropylene based product. Air was subtracted out as the background, and 64 scans were collected.

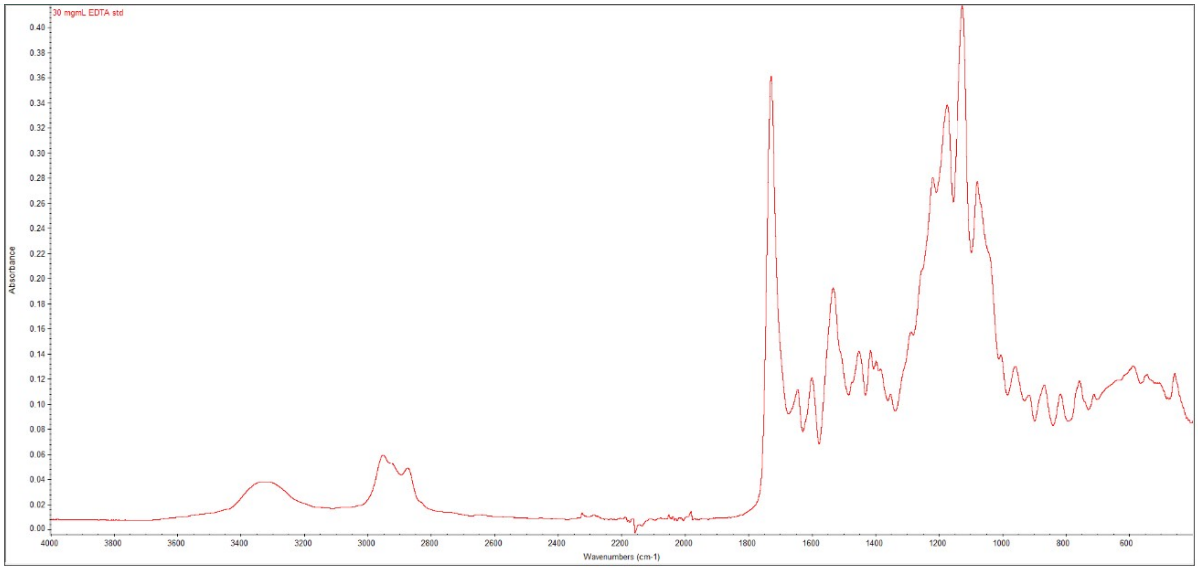


Figure 25: FTIR spectrum of 30 mg/mL EDTA standard in Foam Swab substrate

Above is the FTIR spectrum of the 30 mg/mL EDTA standard deposited onto the foam swab substrate. Air was subtracted out as the background correction, and 64 scans were collected.

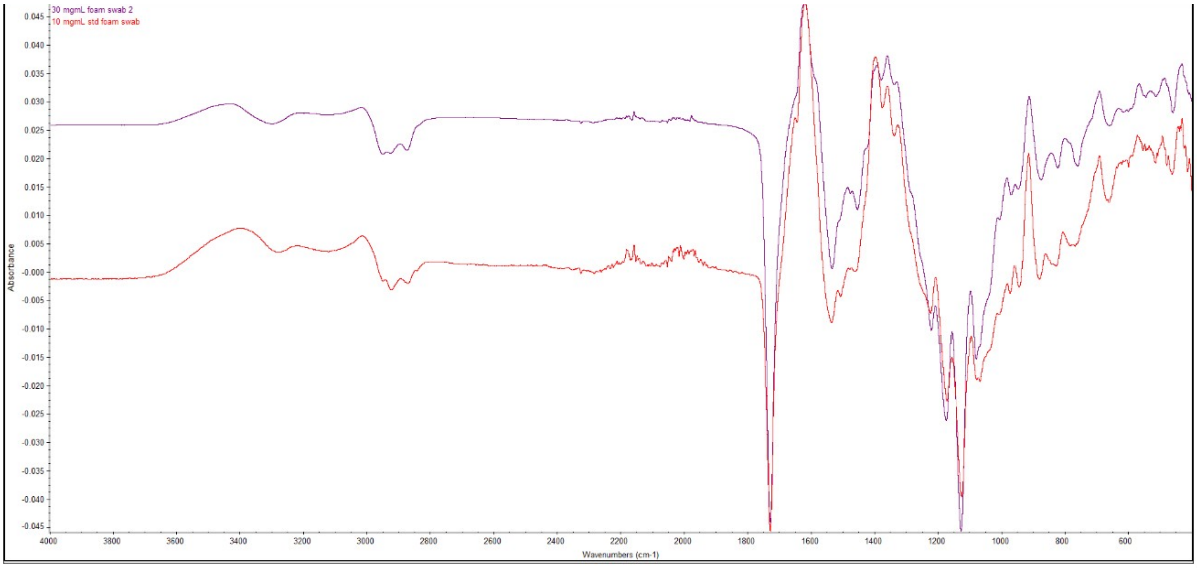


Figure 26: FTIR spectrum of 30 mg/mL and 10 mg/mL EDTA standards in foam swab

Above is the FTIR spectrum of the 30 mg/mL and 10 mg/mL EDTA standard deposited onto the foam swab substrate. The foam swab blank was subtracted out as the background. The spectra were normalized to common scale, and 64 scans were collected.

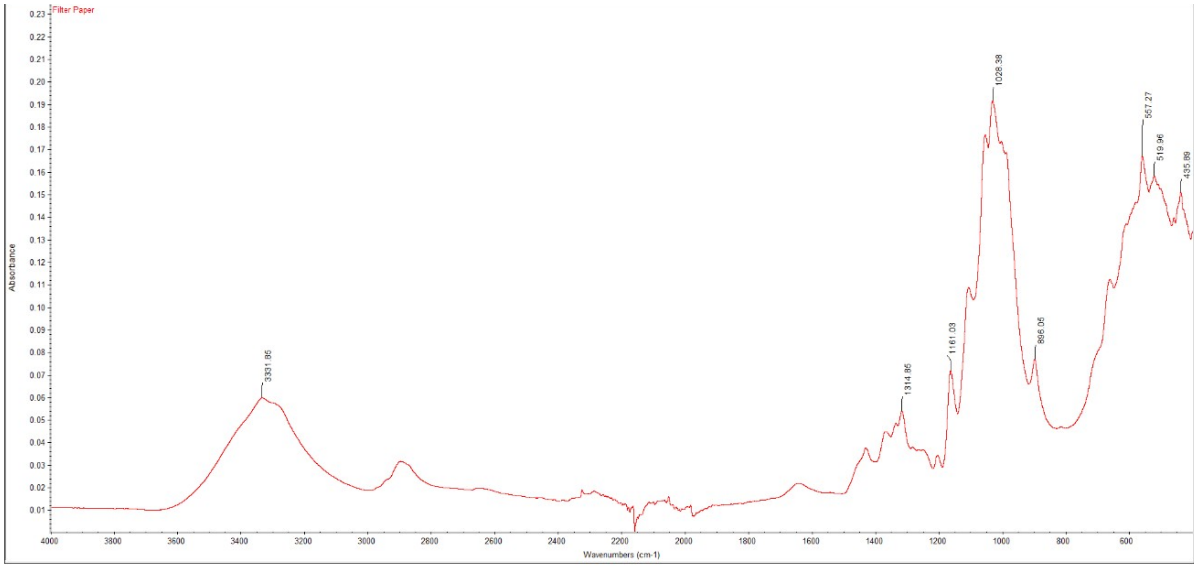


Figure 27: FTIR spectrum of filter paper substrate blank

Above is the FTIR spectrum of the filter substrate blank. Air was subtracted out as the background correction. Overall, 64 scans were collected.

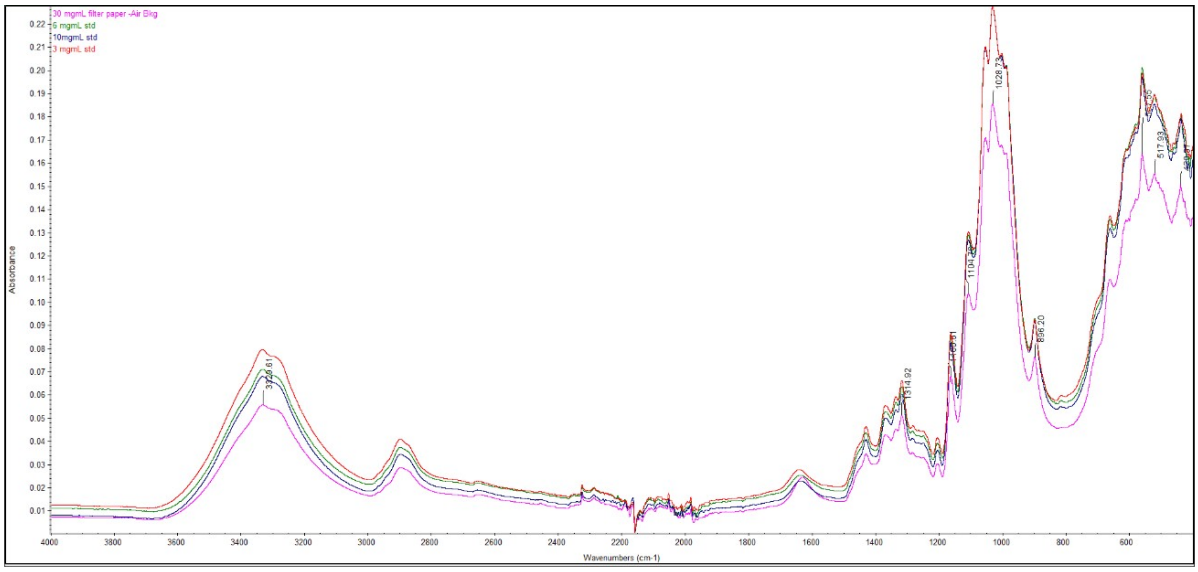


Figure 28: FTIR spectra of 30 mg/mL, 10 mg/mL, 6 mg/mL, 3 mg/mL EDTA standard on filter paper

Above is the FTIR spectrum of the 10 mg/mL, 6 mg/mL, and 3 mg/mL EDTA standard deposited onto the filter paper substrate. Air was subtracted out as the background. A total of 64 scans were collected, and the spectra were normalized to common scale.

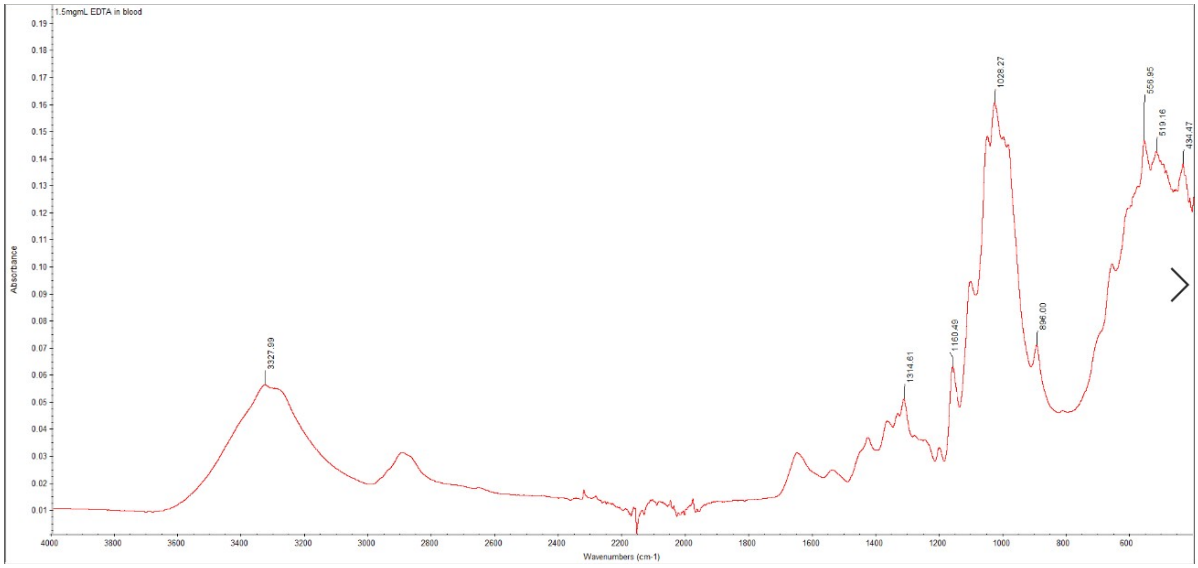


Figure 29: FTIR spectrum of 1.5 mg/mL EDTA in blood sample on filter paper substrate

Above is the FTIR spectrum of the 1.5 mg/mL EDTA in blood sample deposited onto the filter paper substrate. The air was subtracted out as the background. Overall, 64 scans were collected.

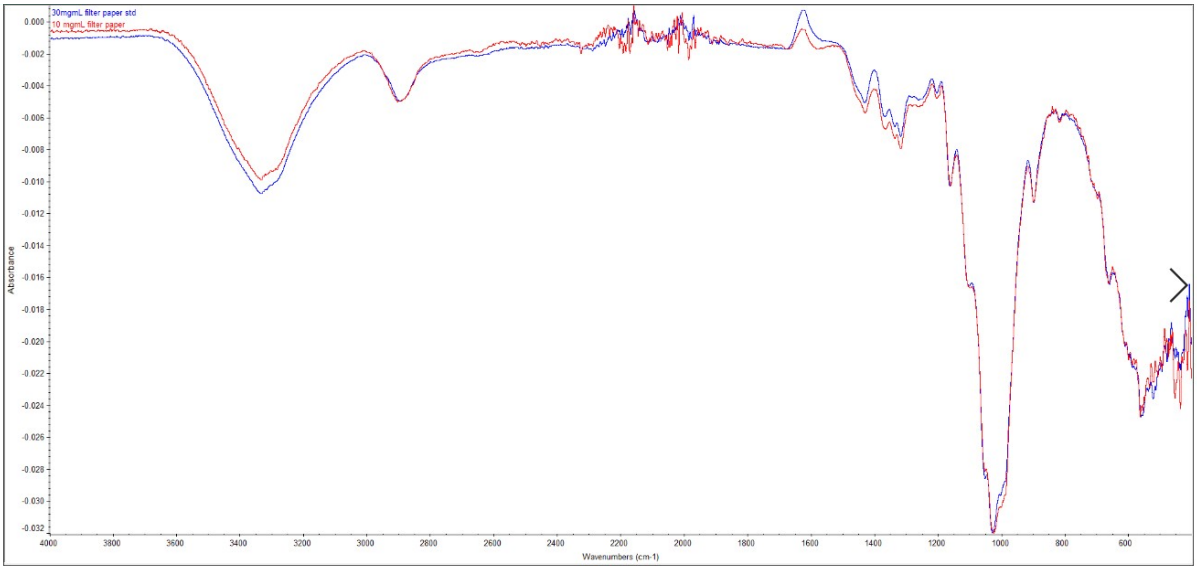


Figure 30: FTIR spectra of 30 mg/mL and 10 mg/mL EDTA standard

Above is the FTIR spectrum of the 30 mg/mL and 10 mg/mL EDTA standard deposited onto the filter paper substrate. The filter paper blank was subtracted out as the background. In total, 64 scans were collected, and the spectra were normalized to common scale.

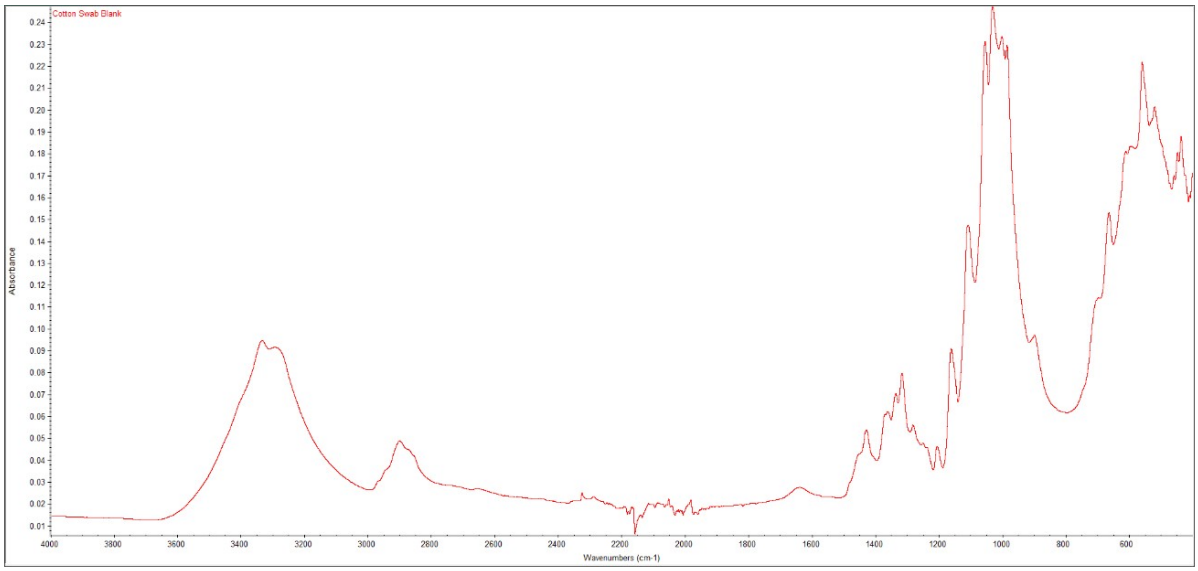


Figure 31: FTIR spectrum of cotton swab substrate blank

Above is the FTIR spectrum of the cotton swab substrate as a blank. Air was subtracted out as the background. The cotton swab is consistent with a cellulose based product. A total of 64 scans were collected.

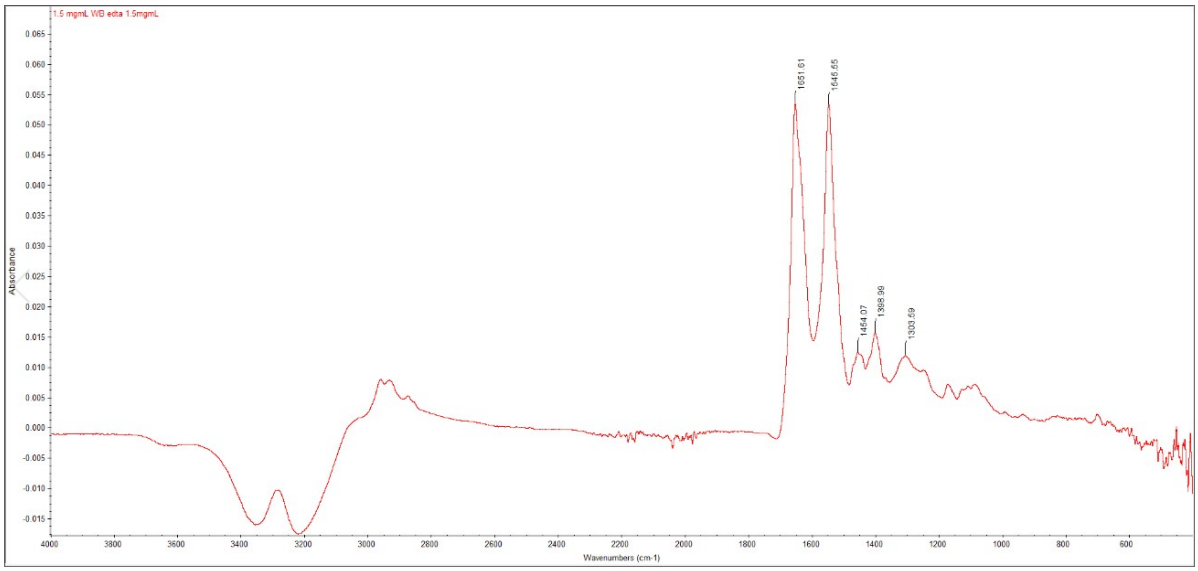


Figure 32: FTIR spectrum of 1.5 mg/mL EDTA in whole blood

Above is the FTIR spectrum of the 1.5 mg/mL EDTA in whole blood. Water was subtracted out as the background. The peaks of interest are at 1651 cm^{-1} and 1545 cm^{-1}. A total of 64 scans were collected.

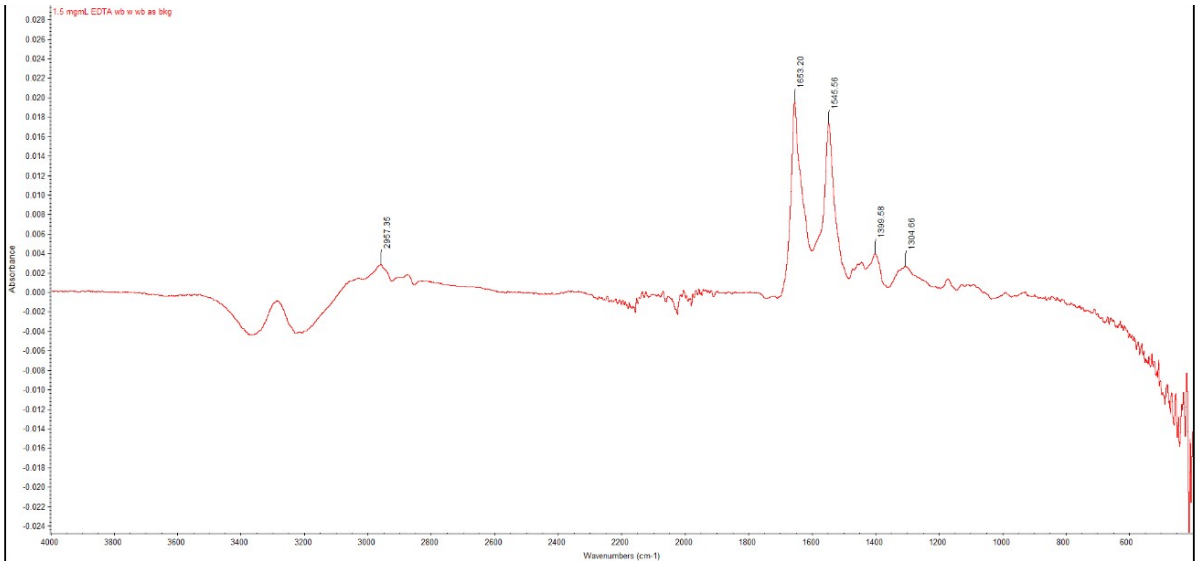


Figure 33: FTIR spectrum of 1.5 mg/mL EDTA in whole blood

Above is the FTIR spectrum of the 1.5 mg/mL EDTA in whole blood. Whole blood without any EDTA was subtracted out as the background. The number of scans collected was 64.

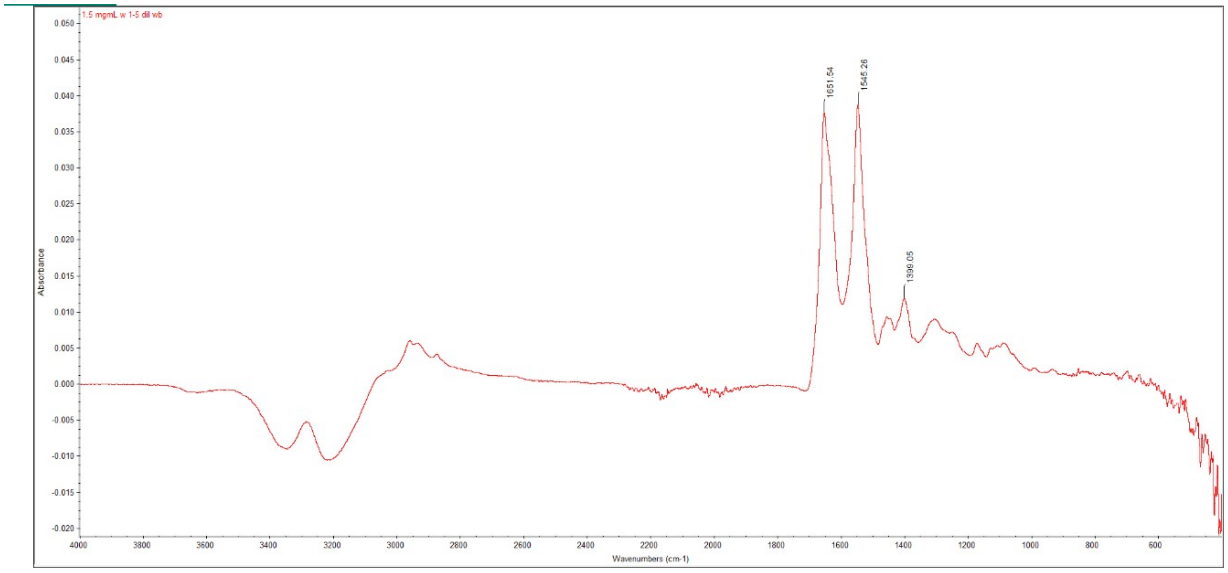


Figure 34: FTIR spectrum of 1.5 mg/mL EDTA in whole blood

Above is the FTIR spectrum of the 1.5 mg/mL EDTA in whole blood. A 1:5 blood dilution was subtracted out as the background. The peaks of interest are at 1651 cm^{-1} and 1545 cm^{-1}. In total, 64 scans were collected.

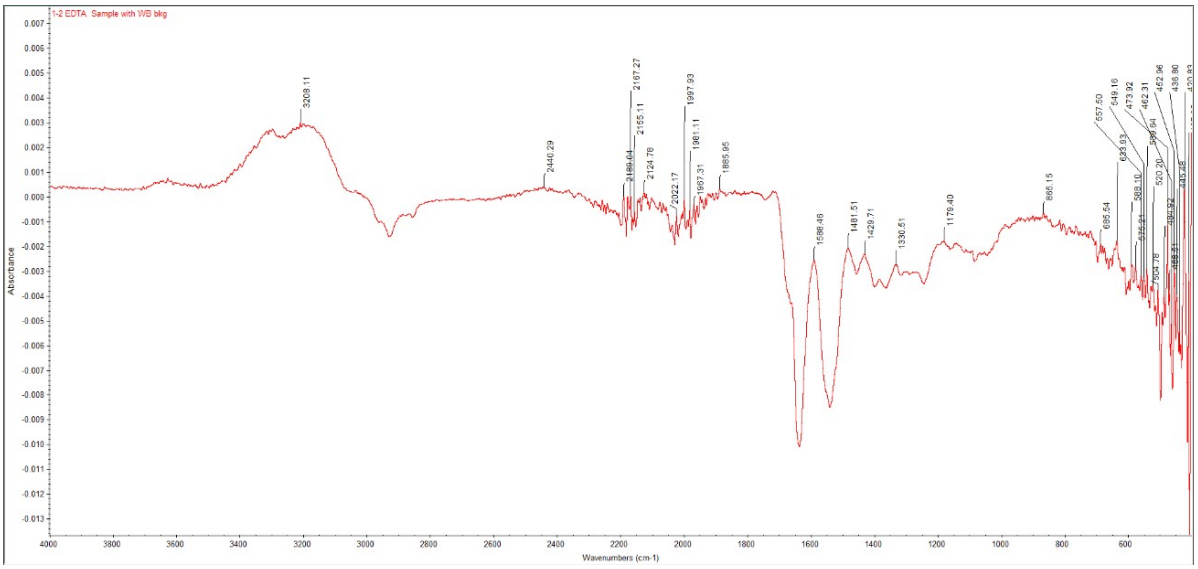


Figure 35: FTIR spectrum of 1:2 whole blood/EDTA dilution

Above is the FTIR spectrum of a 1:2 dilution of the 1.5 mg/mL EDTA in blood sample. Whole blood was subtracted out as the background. A total of 64 scans were collected.

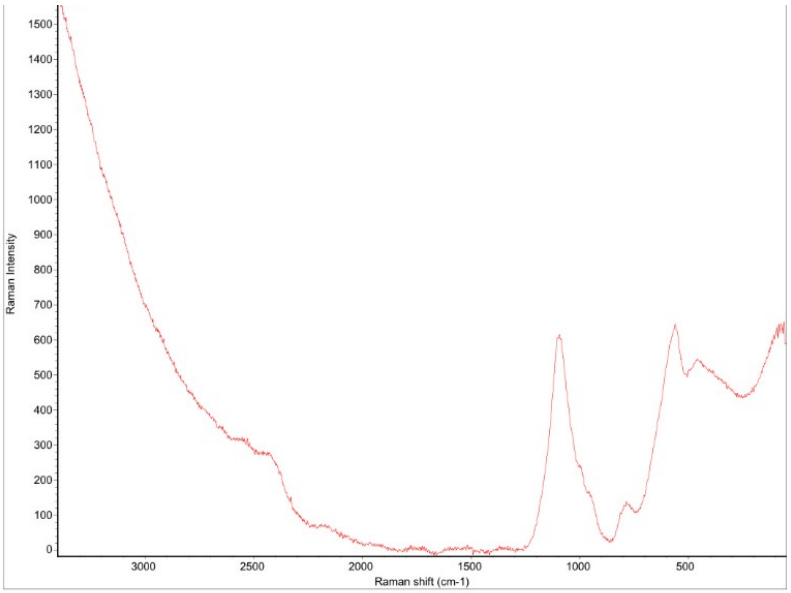


Figure 36: Raman Spectrum of glass slide background

Above is a Raman spectrum of the glass slide background. This method was unsuccessful, as all of the spectra with standard on them looked identical to the glass slide blank.

Table 1 : Signal to Noise (SNR) values for FTIR peaks of interest

Concentration (mg/mL)	1615 cm^{-1} Peak	1401 cm^{-1} Peak
30	3.65	4.30
10	9.26	8.13
6	2.87	2.67
3	3.94	3.40
1.5	2.19	1.58

Above is a table that displays the signal to noise ratios for each of the FTIR peaks of interest, located at 1615 cm^{-1} and 1401 cm^{-1}. The values in question can be reported as a 3.65:1 ratio, when looking at the 1615 cm^{-1} peak value for the 30 mg/mL EDTA standard concentration.

VITA

Alexandra Wright was born in New York City, and raised in Rockland County, NY. Before attending Virginia Commonwealth University, she attended the State University of New York at Albany, where she earned a Bachelor of Science in Chemistry with a Forensics Emphasis. Currently, she is pursuing a Master of Science in Forensic Chemistry-Trace Analysis at Virginia Commonwealth University.