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# Racemization of Amino Acids in Teeth for the Determination of Age

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# RACEMIZATION OF AMINO ACIDS IN TEETH FOR THE DETERMINATION OF AGE

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For my mother: everything I am is everything you made me. You are *always* with me.

To Tshepo for your support, love, and patience without which this would have never been accomplished.

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RACEMIZATION OF AMINO ACIDS IN TEETH FOR THE DETERMINATION OF AGE

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Directed by: Dr. Darwin Dahl, Dr. Eric Conte, and Dr. Kevin Williams

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Instrumental to forensic investigations is the ability to identify unknown human remains providing key evidence to criminal cases, resolution to missing persons, and assistance in mass or natural disasters. Identification of remains in an effort to determine age is an area of forensics that has received considerable attention. Traditional methods in age determination such as morphology are often biased, antiquated, and frequently result in a large margin of error. Conversely, the emergence of new forensic techniques provide promise to reduce the margin of error in determining age. One such technique has focused on relating the extent of amino acid racemization in teeth to age. Past research has focused primarily on the analysis of aspartic acid due to its high racemization rate. Our research indicates that glutamic acid also shows promise as related to age determination. Results will be presented illustrating optimization of gas chromatography using a chiral column for separation of amino acids found in dentin and their enantiomeric ratio quantification. Age correlation data will be presented on collected teeth ranging from mid-teens to early seventies.

## I. Introduction

### A. Background

There is no better judgment of a society than dissecting the fervor in which it pursues truth and justice. According to the Federal Bureau of Investigation's Uniform Crime Report, 13,636 murders occurred in 2009, and currently there are approximately 60 kidnapped or missing persons listed with the FBI alone.[1] While there is not an exact number for unidentified persons that have crossed through coroners' offices around the world, organizations such as *"The Doe Network": International Center for Unidentified and Missing Persons* approximate 4,264 in North America, Australia, and Europe with 3,841 being in the United States.[2] When remains are discovered, it commonly becomes difficult or impossible to determine the person's identity and requires resorting to scientific determinations. Age determination of unknown human bodies is also important in the setting of not only criminal investigations but also mass disasters and missing persons. Establishing the age at death of an individual can aid investigators to the correct identity among a large number of possible matches.[3] Common methods implemented to establish age include morphological, radiological, and instrumental analysis of amino acid racemization.

### B. Morphology

Physical characteristics of bones provide much information about human evolution[4] based on a great number of age-dependent morphological features.[5] In childhood, age estimation can be performed very accurately using developmental methods but in adulthood, the accuracy of most morphological methods is poor.[5] According to Cook et al., regarding the post-40-year age range, anthropologists may only

be able to offer limited statements, such as "mature adult," depending upon which skeletal parameters are available for examination.[6] Also reported is methodological bias and complex variability in the skeletal aging process due to many changes in skeletal form being influenced not only by the chronological age of the individual but also by a number of internal and external factors, such as nutrition, disease, genetic influences[6] and skeletal conditions.[7] Analysis based on morphological methods can yield age estimation error margins of greater than ±10 years.[3] Given the dire importance of correct age identification, a method yielding a more accurate age determination is essential.

C. Radiological

Radiocarbon dating is an analysis used to predict when a person was born based upon the radiocarbon present in the tooth enamel as a result of the nuclear bomb testing during the cold war.[8] While the amount of carbon-14 isotope ($^{14}C$) in the atmosphere remained relatively stable until 1955, above ground nuclear testing caused it to rise dramatically across the globe due to atmospheric equalization.[9] Since 1963 with the institution of the worldwide test ban treaty, the rate of atmospheric $^{14}C$ have decreased with a mean half-life of 16 years thus generating what is known as the "Bomb Curve."[3] Radiocarbon is incorporated into all living things which therefore forms an isotopic chronometer of the past 60 years.[3] In 2005, a novel method was proposed to determine the "bomb" radiocarbon activity in the carbonate component of tooth enamel[6,9] and was followed by an approach to measure the $^{14}C$ in cortical and trabecular bone samples from the same skeleton to establish whether the age range was pre- or post- 1963.[6] Prior to the Second World War, archaeologists had no techniques with which to estimate accurately the age of their finds but can now rely upon $^{14}C$ dating[10] with a margin of error at ±1-3

years[3] within the applicable time frame. Although the method is simple in principle, the level of $^{14}C$ is low and very sensitive radioactive counters are necessary to measure the activity rendering this method as one requiring skilled operators.[10] The need for global availability is crucial and with the limitations presented, a more attainable and functional method for age identification was required.

D. Tooth Development

While methods of dating have been applied to various bones, based on developments in previous research, teeth have been heralded as producing the greatest promise in age identification.[3,6,9] The tooth is comprised of multiple layers and undergoes an intricate development. Figure 1 illustrates the progression of the layers of the tooth from the outer enamel to the internal pulp region. By 1946, knowledge of teeth had improved but still lacked a full understanding. However, fundamental knowledge regarding teeth has remained steady through time. The enamel of the tooth is a very hard structure covering that part which extends above the gum tissue as the tooth crown.[11] Enamel being the hardest tissue of the body[11] comes in contact with the most saliva, mouth debris, and bacteria. Because enamel is the hardest tissue, it is brittle, fracturing easily under stress such as biting or trauma if not supported by the elastic bone-like dentin which forms the bulk of the tooth, crown, and root.[11]

In 1960, the foundations of permanent tooth development were furthered by Carmen Nolla. Nolla's scheme has been referenced recurrently due to the strategic analysis and durable conclusions. In the proposal, normal-developmental curves for each tooth and dental age values for both genders were plotted illustrating no significant differences in sex in the time required for tooth development.[12] Chronologically, Nolla

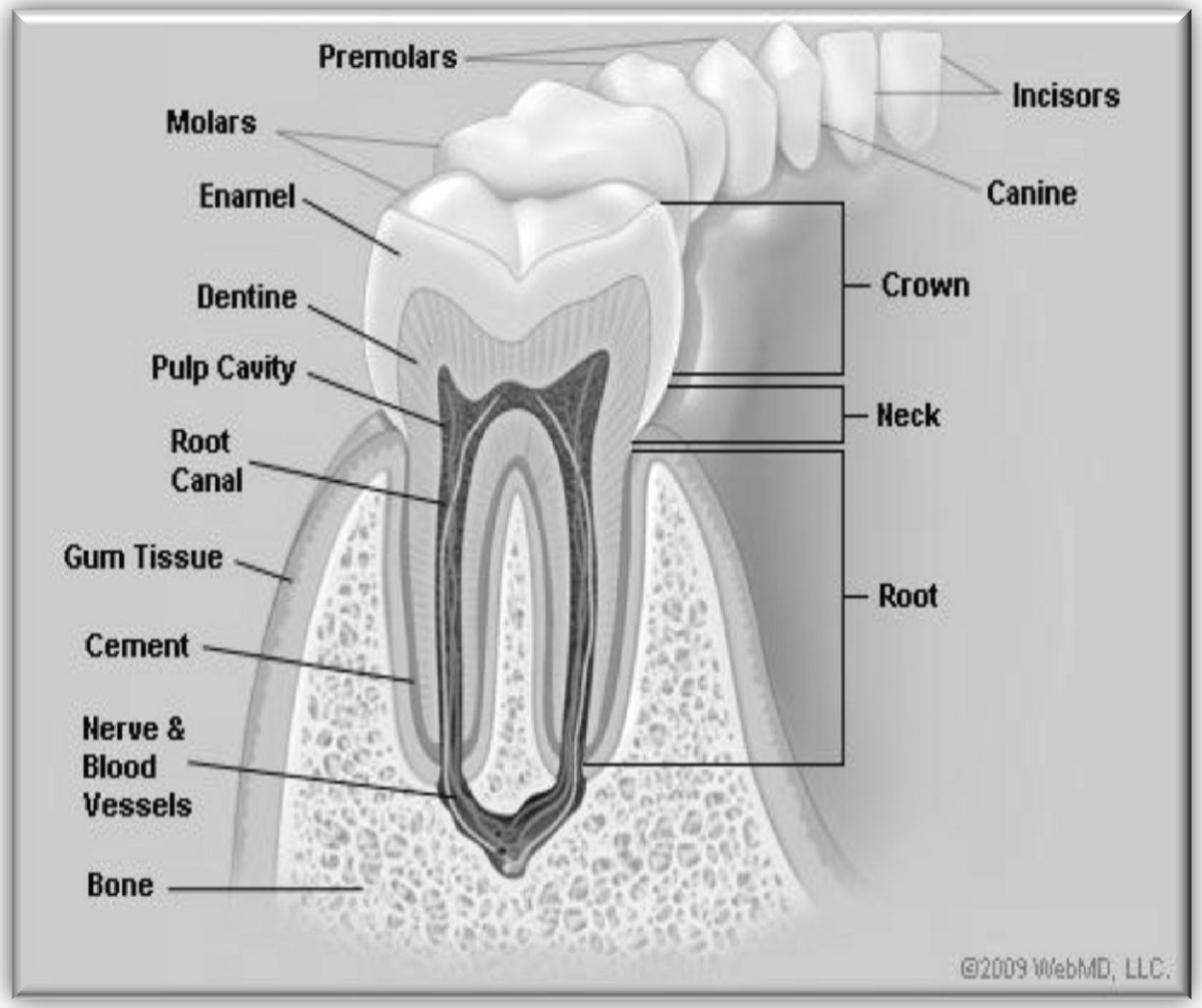


Figure 1: Detailed layers of the tooth illustrating from the outer enamel to the internal pulp region.[36]

proposed a patterned development of teeth. From this pattern, it was demonstrated that teeth do not develop simultaneously from the root of either the mandibular or maxillary teeth.[12] For age determination, the presence of a third molar in either mandibular or maxillary would indicate older age than the central incisor in the permanent teeth. Using the radiographs from teeth over the course of development, age norms were constructed in years based upon the third molar development (due to third molar completion occurring last in both constructs).[12] Also from the data in Nolla's article, given the tabulated values, the assumption is made that the progress in the development of the tooth is a linear function. This discovery carries over time to current researchers who continue to follow the advances made by Nolla and continue to utilize the linear functionality of tooth development.

Further developments regarding the constructs of teeth were made in 1976 when Helfman and Bada delved deeper into the proteinaceous composition found in the different layers of the tooth. Their studies indicated various organic matrices in the enamel and throughout the crown but determined that dentin accounts for a much greater proportion of the tooth with dentin being 100 times greater in protein composition than that of any other portion.[13]

E. Amino Acid Racemization

Over time, various amino acids have been discovered in teeth. The L-amino acids initially present in bone protein undergo slow racemization over geological time at a rate which is proportional to temperature.[13] However, a system containing exclusively L-amino acids is thermodynamically unstable, since under conditions of chemical equilibrium, D- and L-amino acids are present in equal amounts.[14] At the human body

temperature of approximately 37 °C, amino acid residues in tooth enamel protein undergo racemization from L- to D-residues at a rate of approximately 0.1% per year.[13] Racemization is a natural process which will eventually convert optically active compounds into a racemic mixture but would take about 100,000 years at 25 °C for all L-amino acids present in living systems to undergo complete racemization to an equilibrium mixture.[15] Figure 2 illustrates the general conformational change from the L- to intermediate to the D-conformation.

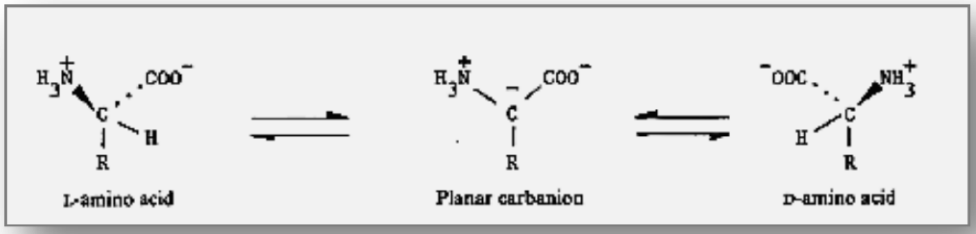


Figure 2. General conformational change of an amino acid from L-amino acid with an intermediary step to the D-amino acid.[14]

In 1932, du Vigneaud assessed amino acids for conditions, ease, and totality of racemization to extend a previous study he completed to other representative amino acids in detail.[16] du Vigneaud illustrated racemization of amino acids with sodium salt in aqueous solutions with acetic anhydride as well as concluding that certain representative amino acids such as glutamic acid can be completely racemized in this way.[16] He reported determining the rate of racemization in his reaction for acetylglutamic acid while noting that no racemization occurred with proline.[16] du Vingneaud's work was a precursor to the development of methods that focus on the conditions necessary to illicit racemization. On this basis, future research has been sought to perform derivatization of

the amino acids without causing chemically induced racemization which would affect the analysis of teeth for age determination.

The bones of living animals contain about 25-30% organic material comprised primarily of proteins.[14] Concerns have been voiced regarding amino acid racemization data since the amino acids in the various hydrolysis products have been found to have different racemization rates.[14] Bada addresses these concerns in 1985 by studying the kinetics of amino acid racemization using laboratory simulation experiments and dated fossils collected in environments similar to "present-day" temperatures. He reports finding the conversion of the D/L ratios measured are only as reliable as the calibration curve that was used to make the determination.[14] Bada also discovered that elevated temperatures show a pattern of D- to L- conversion during racemization which he states provides the ability to calculate the racemization age of a fossil.

F. Aspartic Acid Racemization

Of the various amino acids that have been found in teeth, aspartic acid has received the highest amount of attention from researchers. Most report focus on aspartic acid due to its stability[15] but more so for the rate in which racemization occurs.[13,14,15] Helfman proposed in 1976 that the reaction is irreversible as well as first-order yielding the ability to utilize a least squares fit to produce a calibration graph.[13] However, in 1985 it was determined that racemization is a reversible first order rate process[17] and the rate of accumulation of D-aspartic acid is generally greater than those of other amino acids.[18] While Ohtani reported in 1997 the aspartic acid D/L ratio in dentin "correlates extremely well with age,"[19] questions arose regarding the derivatization procedure of the amino acids[20] as well as atypical kinetics.[21] Collins reports these atypical patterns have proved

more difficult to describe mathematically than other amino acids but goes on to report the D:L ratio transformations are useful in calibrated investigations to assess age due to a strong correlation with time.[21] A further speculation was made regarding aspartic acid kinetic rates. In 2002, Poinar reported the rate of racemization of amino acids depends predominantly upon the electron-withdrawing capabilities of the R substituents, namely the more electron-withdrawing the R group, the faster the rate of racemization.[22] By 2005, it was accepted that the relationship between the degree of racemization in teeth showed a high degree of correlation to age and the established racemization method possessed a high degree of reliability.[3,23,24] Likewise, by 2007 the degree of aspartic acid racemization in dentin was considered to be one of the most accurate methods for the estimation of chronological age[25] with accepted kinetics as a reversible temperature dependent reaction.[3,24,25,26,27]

G. Expanding the Amino Acid Scope

While aspartic acid has been heralded since 1976, other amino acids are present in the tooth that offer promise as age identification markers. Alanine, serine, aspartic acid, and glutamic acid are nonessential chiral centered amino acids which are produced in the human body through diverse pathways of biosynthesis.[28] Nonessential amino acids have a common feature in that their carbon skeletons come from intermediates of glycolysis, the pentose phosphate pathway, or the citric acid cycle allowing them to be synthesized in simple to complex reactions.[28] The chirality of the amino acid formed is determined by the direction from which a proton is added to the quinonoid (proton loss during Schiff-base linkage during the beginning stages of synthesis).[28] These commonalities led to the

conjecture that additional amino acids present in teeth would also yield a possible correlation to chronological age.

Glutamic Acid is a dibasic acid that du Vagneaud evaluated in 1932 to determine its racemization and optical rotation. His results yielded an important springboard for the analysis of glutamic acid when he reported that racemization in his experiment proceeded rapidly at first and then began to gradually decrease.[16] This experiment was conducted to determine the amount of "fractionation" that was occurring in the amino acids. Researchers report aspartic acid is an inaccurate age determinant because it is actually the combined response of aspartic acid and asparagine due to asparagine's decomposition to aspartic acid upon acid hydrolysis.[21,24,29] This would indicate the accuracy of aspartic acid as an age determinant is questionable unless the appropriate acid hydrolysis occurred. The fractionation of the aspartic acid with asparagine residues presents possible issues even though researchers have found high correlation using only aspartic acid.[13,19,23,25,27,30,31,32] In the case of glutamic acid, based upon the rotation that was observed by du Vigneaud, he reports practically no fractionation occurred.[16]

Serine can be thought of as a version of alanine with a hydroxyl group attached making serine much more hydrophilic and reactive than alanine.[28] Like aspartic acid, the useful nature of serine as an age determinant has been questionable.[24,33] However, others have found serine as a promising amino acid to be used as an age determining marker.[23,24,26,34] Proteins housing the amino acids can be subcategorized into soluble and insoluble fractions in which racemization in the total protein has been reported as reliable for making age estimates.[23,29] Similarly, dentin also has these same subcategories in which collagen, the major contributing protein to D/L ratios,[23] also has soluble and

insoluble components.[29] According to Waite, higher solubility yields the fastest racemizing components and states it is potentially the most accurate.[29] In 2000, Cloos reported on amino acids when phosphoproteins and collagen from an age series of human dentin were observed.[34] He described serine as having a complex change, and like that found in glutamic acid,[16] initially increased quickly but reports reaching a plateau after approximately 40 years.[34] Cloos demonstrated in his study that serine measurements found in his experiment could be applied in forensic science for age-at-death determination but that additional studies were required to add further biological significance to the data.[34] In 2008, serine had not attained the same age determinant status as aspartic acid but continues to be suggested that greater emphasis on serine would improve the age estimates that are produced.[24]

Although alanine is present in tooth dentin with easily separated D- and L-enantiomers,[23] much focus has not been placed on the viability of this amino acid as an age determinant marker. Arany established one of the few regression analyses for alanine finding the calculated standard error for age determination was ±2.31 years.[26] However, while an age calibration curve was generated and correlations made, Arany contests that alanine racemization has not gained affirmation for age estimation attributable to difficulties in explicit quantification of the D-conformation from dental samples and un-resolved chromatographic peaks.[26] Arany concludes regarding alanine much like Cloos regarding serine[34] in that given refined separation, implications exists to facilitate the design of future amino acid racemization studies for further analysis.[26]

H. Goals of the Research

Previous research has detailed various amino acids as age determining markers but rendered many questions unanswered. Others have sought to establish derivatization procedures or a standard protocol for the isolation of the amino acids.[13,15,17,19,21,29,30,35] This research aims to establish an optimal method for amino acid derivatization using tooth dentin as the vehicle. Likewise, this research will evaluate the disputed amino acids alanine, serine, aspartic acid, and glutamic acid in an effort to establish age correlation using the D- to L- ratios produced by gas chromatography methodology.

## II. Experimental

### A. Chemicals and Materials

Racemic amino acid standards were purchased from Sigma-Aldrich: D-alanine (≥99% TLC), L-alanine (≥98% TLC), DL-alanine (≥99% FCC), D-serine (≥98% TLC), L-serine (≥99% TLC Reagent Plus®), DL-serine (≥98% TLC), D-aspartic acid (99%), L-aspartic acid (≥98%), DL-aspartic acid (≥99% TLC), D-glutamic acid (≥99% TLC), L-glutamic acid (≥99% TLC Reagent Plus®), DL-glutamic acid (≥98% TLC). HPLC grade isopropanol was purchased from Fischer Scientific while ECD grade acetone and dichloromethane were purchased from Acros Organics. HPLC grade methanol was purchased from Honeywell while 2-butanol (≥99% Reagent Plus®), trifluoroacetic anhydride (≥99% Reagent Plus®), acetyl chloride (98% Reagent Plus®), and Dowex 50W-X8 50-100 mesh was purchased from Sigma-Aldrich. Ethanol was 200 proof and ethyl acetate (≥99%) was obtained from Sigma-Aldrich. Compressed nitrogen, chromatographic grade compressed helium, breathing quality compressed air, and compressed hydrogen were purchased from Airgas. A Barnstead NANOpure II filtration system provided the 16.7 megaohom-cm water used throughout the experiments as is referred to as "filtered water."

### B. Instrumentation

#### B.1. Gas Chromatography

Analysis was conducted using a Hewlett Packard 5890 Series II Gas Chromatography equipped with an Agilent 3396 Series III integrator. Helium functioned as the carrier gas and nitrogen, air, and hydrogen fueled the flame ionization detector. The injector port was outfitted with a Thermogreen LB-2 septa and 1 μL manual

injections completed with a 10 μL Hamilton gas tight syringe. Gas chromatography specifications began at 80 °C for 15 minutes followed by a 2 °C/minute temperature ramp to a final temperature of 135 °C. The injector temperature was set to 280 °C and the FID detector was set to 300 °C. Integrator specifications for this research were as follows: threshold (0), attenuation 2↑(1), area rejection (500), and a run time (55 minutes). The GC was outfitted with a Chirasil-L-Val column (25 m X 0.25 mm internal diameter) which carries a maximum temperature of 200 °C.

B.2. Light Microscope

Light microscope pictures were taken using a JVC, KY-F75U (10X2.0X) with the KL 1500 LCD light set to 3200K. Compilations of the images were completed using auto montage software.

B.3. SEM-EDX

Scanning Electron Microscopy – Energy Dispersive X-Ray Spectroscopy was completed using a JEOL JSM-5400 LV model instrument. Samples were placed into the instrument using Ted Pella 16073 double coated carbon conductive tape (8 mm width x 20 m length). The scope was set at 20 kv with a load current of 56 μA. The spot size was set to one o'clock in aperture 2 in the BEI position I on low vacuum mode of 130 mtorr.

C. Esterification Alcohol for the Amino Acid Standards

Previous research has shown variability in the choice of the Esterification alcohol, and based upon this inconsistency the derivatization procedure required modification. Four alcohols were selected in this study with regards to resolution and recovery.

Previous alcohols employed were methanol and isopropanol. In addition this study evaluates ethanol and 2-butanol as esterification alcohols as well.

The four amino acids selected for study were alanine, serine, aspartic acid, and glutamic acid. The four racemic amino acid standards were combined using 1.000 g of each and placed in a mortar. The standards were mixed until homogeneity was likely achieved and then placed in a 100 °C oven for 15 minutes to dry. Once dry, the sample was ground by mortar and pestle for approximately five minutes. For derivatization, four 5.0 mg portions of the standard were weighed into a 5 mL Wheaton® serum vial. Unused dried standards were placed in a weighing bottle with a standard taper joint and housed in a desiccator.

The esterification step involved reacting a 20:1 v/v mixture of the selected alcohol to acetyl chloride. The alcohol was placed in a 50 mL beaker while chilling in ice. Acetyl chloride was then added drop-wise while stirring to the chilled beaker of alcohol. Two milliliters were extracted from the beaker and pipetted into the serum vial containing 5.0 mg of the amino acid standard. The contents of the vial were gently swirled and sealed with a 20 mm natural rubber serum cap. An aluminum crimp was then utilized to seal the serum cap to the vial. The sealed vials were placed in the oven at 70 °C for 30 minutes.

Following this period, the vials were allowed to return to room temperature for safe handling. The aluminum and serum caps were removed. The vial was placed on a Brinkman R110 rotating evaporator at 45 °C until a resultant yellow-red oil remained. The sample was removed from the Rotovap and dried to completion using nitrogen gas. One milliliter of dichloromethane was subsequently placed in the vial and swirled to

dissolve the residue. The sample was again evaporated using nitrogen gas until the solvent was depleted and dried oil remained.

The final step of the derivatization procedure required acetylation. This involved the addition of 1 mL of trifluoroacetic anhydride to the dried oil residue. The vials were sealed with serum stoppers and capped with aluminum coverings. The vial was placed in the oven for 15 minutes at 90 °C. After treatment, the vials were removed and the contents were transferred into a 1 mL conical vial. The mixture was evaporated using nitrogen gas. Once dry, 1 mL of dichloromethane was added to reconstitute the sample. The sample was then ready for analysis by gas chromatography.

D. Esterification Alcohols for the Tooth Dentin

While the standards provide data regarding the amino acids, it was also imperative to perform this alcohol analysis on the teeth samples given the more robust matrix. Therefore four-10 mg samples of tooth dentin were weighed in a Wheaton® 10 mL serum vial and 10 mL of 6 M HCl was added. Hydrolysis with HCl was performed for 6 hours at 90 °C. The samples were transferred to a 25 mL round bottom flask and evaporated to a dry solid using a Rotovap. Next, 5 mL of nanopure water was added and placed on a cation exchange resin column detailed in section VII. Resin Column Volume and Isolation. The amino acids were eluted using aqueous ammonia and evaporated to a dry solid. The esterification process was completed using one alcohol per sample using the methodology of the standards. Following the esterification, the acetylation step proceeded in the same manner with the exception of a reconstitution volume of 100 μL dichloromethane. Gas chromatography was then completed once the derivatization process was concluded.

E. Gas Chromatographic Parameters

Gas Chromatography analysis was performed on an HP 5890 Series II instrument equipped with an Agilent® CP-Chirasil-L-Val 20 m column and a flame ionization detector (FID). Chromatograms were produced using an Agilent® 3396 Series III integrator with Helium as the carrier gas. Nitrogen, breathing quality air, and hydrogen gasses flowed into the FID. Initial temperature was set at 80 °C for 15 minutes followed by a 2 °C/minute temperature ramp to a final temperature of 135 °C. Run time was set to 55 minutes. One microliter manual injections were completed using a Hamilton® 10 μL gas tight syringe. The syringe was cleaned before and after sample injections using dichloromethane. Prior to injection, the needle of the syringe was cleaned using a KimWipe to remove any contamination upon injection. The injector temperature was maintained at 280 °C with the FID set at 300 °C.

Optimal parameters for the Gas Chromatography analysis were achieved through a range of variations of temperature, time, and pressure. Previous research established parameters that were used as guidelines for temperature programming. To determine the optimum head pressure, a standard amino acid sample prepared under the methodology detailed in section I. Esterification Alcohol was analyzed. A standard amino acid sample was analyzed by the described GC condition at the following head pressures: 17.5 psi, 18.0 psi, 18.5 psi, and 19.5 psi. The enantiomeric peaks were measured to determine resolution, peak broadening, and analysis time. The optimum head pressure was determined to be 18.5 psi.

Once the optimum head pressure was established, an optimum temperature ramping program establishment was necessary. Alanine, being the lightest amino acid

was of initial interest and therefore needed to be adequately separated from the solvent line. This separation was dependent upon the initial program temperature. The initial temperatures investigated ranged from 75-100 °C at 5 °C increments. Alanine at a concentration of 5 mg/mL was used to determine the degree of its separation from the solvent front.

A further determination for optimal GC parameters included the hold time. This is an important parameter in the successful separation of larger amino acids. To ensure adequate baseline resolution throughout, the hold time was varied to include 10 minutes, 12 minutes, and 15 minutes for the analysis of the mixed amino acid standard. The optimal temperature programming was established to be a 15 minute hold at 80 °C followed by 2 °C/ minute temperature ramp to a final temperature of 135 °C.

F. Preparing the Tooth for Analysis

In preparation for the analysis of the teeth samples, removal of outside contamination is essential and accomplished by performing a series of washes. In a 50 mL beaker, the tooth was submerged in a 6 M hydrochloric acid solution and ultrasonicated for five minutes. The HCl was decanted and replaced with filtered water that had been filtered through the Barnstead NANOpure II filtration system and sonicated for five minutes. The Nano pure water was also decanted and replaced twice more with Nano pure water, sonicating for five minutes each time. Ethanol was used as the third rinse solvent and ultrasonciated for five minutes. Following this, a final step of submersion in ethyl acetate was performed without sonication for five minutes. The tooth was removed from the ethyl acetate and allowed to dry before being placed under glass wool in a glass 5 mL vial with a snap cap.

G. SEM-EDX

Longitudinal cuts were made with a Dremel drill using a diamond blade which exposed the interior layers of the tooth. To identify the more proteinaceous material, Scanning Electron Microscopy with Energy Dispersive X-ray spectroscopy was performed. The tooth was placed cut side up using double coated, 8 mm(w) x 20 mm(l) carbon conductive tape on a silver plate. Low vacuum was achieved at 130 mtorr in a JEOL JSM-5400 LV Scanning Microscope and analysis conducted on sections of the tooth using an overhead beam of electrons. The outer enamel layer was analyzed first with successive inward analyses conducted until the interior pulp region was reached. The scope was set at 20 kv with a work load current 56 μA. The spot size was at 1 o'clock in the BEI position 1. More intense carbonaceous material was found in the dentin locus of the tooth as determined by EDX and therefore identified for future extraction.

H. Cutting the Teeth

Past researchers employed methods requiring pulverization followed by grinding to a fine powder by mortar and pestle. This technique inherently results in a higher concentration of enamel obtained. In order to reproduce these results, a longitudinal cut was made from the crown of the tooth to the root using a Dremel drill with a diamond blade. Once the interior was exposed, additional cuts were made to isolate the dentin by carving away the layers of enamel. The isolated dentin was placed in a mortar and pestle and ground to a course powder. Isolating the dentin in this manner was inefficient due to larger amounts of enamel contaminating the sample as well as an inability to fully grind the robust dentin into a manageable powder.

To ameliorate this issue, a new technique was employed in which the tooth was no longer cut longitudinally. Rather, a thin layer of the crown was removed taking special care the concavities containing the enamel were removed. The diamond blade was then exchanged for a diamond tip drill bit. The dentin was excised by immediate pulverization as the tip hollowed the interior of the dentin core. Use of the diamond drill bit yielded an instant powder of the dentin which required no further grinding. Careful control of the placement of the bit allowed almost predominantly dentin to be removed.

I. Acid Hydrolysis of Tooth Dentin

Ten milligrams of dentin were weighed in a 10 mL Wheaton® serum vial. Ten milliliters of 6 M HCl acid was pipetted into the vial containing the dentin and sealed with a 20 mm natural rubber serum cap. The seal was then secured with an aluminum crimp and placed in the oven for 6 hours at 90 °C. Once six hours had expired, the vial was removed from the oven and allowed to cool to room temperature. The seal was removed and the solution transferred to a 25 mL round bottom flask outfitted with a microscale connector 20-400 x 20-400. The solution was then evaporated using a Brinkman R110 Rotovap until the resulting product was a yellow solid. Five milliliters of filtered water was added to the round bottom flask to dissolve the amino acids.

J. Resin Column: Volume and Isolation

After acid hydrolysis of the sample, the isolation phase commenced in which the amino acids were sequestered from the complex matrix. This is achieved using a Sigma Aldrich® Dowex 50W-X8 cation exchange resin 50-100 mesh.

To effectively isolate the amino acids and remove impurities, the optimum volume of resin used needed to be determined. To make this determination, resin

volumes were varied and the amino acid recovery was determined. A 50 mL volumetric buret with stopcock was cut to approximately 25 mL to create a column for the resin. Using a graduated cylinder, measured volumes of 1, 2, 3, and 4 mL of resin were placed individually in the buret for analysis. To initially prepare the column, ten milliliters of 1.0 M HCl was placed in the cylinder and transferred to the column. The stopcock was opened to allow elution at the rate of 1.9 mL/minute. One milliliter of filtered water was added to the column as the volume reduced to approximately 1 mL above the top of the resin. This was conducted until 30 mL of water had been added. Next, 5 mL of 2 M sodium hydroxide was added in 1 mL additions. Twenty milliliters of water followed the NaOH in 1 mL additions. To re-acidify the column, 10 mL of 6 M HCl was added to the column and was followed by 30 mL successive nanopure washes. Following the final water treatment, the amino acid standard prepared in section C. Esterification Alcohol for the Amino Acid Standards, previously divided into four-5 mg samples, was placed in individual 25 mL round bottom flasks. Five milliliters of filtered water was added to each round bottom flask and transferred to the designated resin column. The aqueous layer was allowed to elute from the column until approximately 1 mL above the resin. Ten milliliters of filtered water was then added and allowed to drain to just above the resin column. Using 2 M aqueous ammonia, the amino acids were then eluted from the resin column using a volume of 10 mL aqueous ammonia at a rate of 1.9 mL/min and collected in a clean, dry 25 mL round bottom flask. The resin heights were measured to ascertain the volume of the resin in the column. The round bottom flask was then outfitted with the microscale connector and evaporated using a Rotovap. To ensure complete evaporation, a nitrogen gas stream was used to complete drying of the sample.

As a control, one amino acid sample from the same stock received no treatment on the resin column and experienced only the derivatization procedure. The samples eluted from the four various resin column volumes also underwent the derivatization procedure after evaporation. The samples were then analyzed using gas chromatography in which the percent recovery was determined.

It was determined a resin column volume between 2 cm and 3 cm was optimal and therefore applied to the dentin samples. Following acid hydrolysis, a dentin sample was added to the resin column as per the procedure of the standards. Once evaporated, the dentin samples underwent esterification and acetylation steps detailed in section M. Dentin Analysis. The dentin samples were then analyzed using gas chromatography.

K. Ruggedness

Given the length of the procedure, samples were often unable to undergo GC analysis until the following day. To ensure the integrity of the samples, a ruggedness test was conducted. Five milligrams of the standard amino acid prepared in section C. Esterification Alcohol for the Amino Acid Standards underwent the esterification and acetylation process. The standard sample was analyzed over a three week time frame under identical GC conditions. The length of time between analyses averaged every three days. The percent recovery was determined and graphed over time interval.

L. Reproducibility

To establish validity of the method, dentin samples were analyzed for reproducibility based upon relative standard deviation. Dentin from a single tooth was divided into four-10 mg samples. These samples experienced acid hydrolysis, isolation, elution, esterification, and acetylation with a reconstitution volume of 100 μL of

dichloromethane. The samples were then analyzed by GC to determine the ratio of the amino acid peak heights and the relative standard deviation among the four samples were calculated.

M. Dentin Analysis

Once the methodology had been established and evaluated for optimal conditions, a collection of dentin samples were analyzed for the establishment of an age determinant calibration curve.

Ten milligrams of tooth dentin was weighed into a 10 mL Wheaton® serum vial and 10 mL 6 M HCl was added. The vial was sealed using a 20 mm natural red rubber stopper and sealed using a 20 mm aluminum crimp. The sample was placed in the oven at 90 °C for 6 hours. Once 6 hours had expired, the sample was allowed to come to room temperature and then transferred to a 25 mL round bottom flask outfitted with a microscale connector. The solution was then evaporated using the Brinkman R110 rotovap until a yellow solid remained. Five milliliters of filtered water was added to the flask to dissolve the solid. A Dowex 50W-X8 50-100 mesh cation exchange resin column was utilized to isolate the amino acids from the dentin matrix. Application of the column detailed in section J. Resin Column: Volume and Isolation was performed with a final elution using 2 M aqueous ammonia. The solution was again placed on the rotovap for evaporation until no liquid solution remained.

A 20:1 ethanol to acetyl chloride solution was prepared in an ice chilled beaker and adding acetyl chloride drop-wise to the stirring solution. Two milliliters of the ethanolic HCl was placed in the round bottom flask and then swirled for collection. The solution was transferred to a 5 mL serum vial, sealed, and crimp capped. The vial was

placed in the oven at 70 °C for 30 minutes and subsequently evaporated until a yellow-red oil remained. One milliliter of dichloromethane was added to the solution, swirled, and evaporated using nitrogen gas. Following this esterification step, acetylation commenced with the addition of 1 mL of trifluoroacetic anhydride. The serum vial was then sealed, capped, and placed in the oven at 90 °C for 15 minutes. Once time had expired, the solution was transferred to a 1 mL conical vial and evaporated using a stream nitrogen gas. One hundred microliters of dichloromethane was added to the conical vial, sealed with a Teflon cap, gently mixed, and finally transferred to a 2 mL GC vial outfitted with a 200 μL glass volume raise. One microliter volumes of each sample were injected into the GC at the established parameters.

## III. Results and Discussion

### A. Esterification Alcohol

Using an amino acid standard containing D/L alanine, D/L serine, D/L aspartic acid, and D/L glutamic acid, four samples underwent the derivatization procedure using a variation of alcohols. Sample one received methanol, sample two received ethanol, sample three received isopropanol, and sample four received 2-butanol as the esterification alcohol. In order to select the most effective alcohol for this step resolution was evaluated. Ideally, baseline resolution between peaks occurs between 1.2 and 1.5.[37] The alcohol chosen to be optimum was determined based upon two factors: baseline resolution over 1.2 and the retention times of the amino acids. Resolution was calculated using Equation 1.

*Equation 1:*

$$Resolution = \frac{(T_2 - T_1)}{\left(\frac{1}{2}\right)(W_1 + W_2)}$$

where:

| | |
|---|---|
| $T_2$ = | Retention Time L-Amino Acid (min) |
| $T_1$ = | Retention Time D-Amino Acid (min) |
| $W_1$ = | Measured Base Width D-Amino Acid (cm) |
| $W_2$ = | Measured Base Width L-Amino Acid (cm) |

Centimeters were converted to minutes by using the chart speed: 0.4 cm/min.

B. Alcohol Standards

Methanol and isopropanol were used as esterification agents in the experiments due to reports of success in previous research.[19,26,35] We chose to look at extending the scope of alcohols used to optimize the amino acid separation. In this work, we included ethanol and 2-butanol as esterification alcohols. The use of ethanol and 2-butanol is possibly novel in its approach due to no current findings of their usage as esterification alcohols for amino acid derivatization by this researcher. The primary objective of this study was to determine an effective esterification alcohol based on retention time, recovery, and resolution.

C. Distinguishing the Peaks

Prior to calculating resolution of the peaks, retention times of the amino acid peaks must be determined. Amino acid standards were derivatized and chromatographed individually to determine the retention of the D- and L-peaks. Figures 3 and 4 show aspartic acid's respective chromatograms. The four samples receiving treatment with the varying esterification alcohols were chromatographed and the amino acid peaks identified (Figures: 5, 6, 7, and 8). Distinguishability from baseline noise was essential to identify the peaks with accuracy. Chromatograms produced utilizing isopropanol and 2-butanol as the esterification alcohol proved problematic. Using isopropanol, glutamic acid peaks were difficult to assess due to multiple peaks being present. Likewise, the identity of aspartic acid was problematic but not unattainable due to multiple peaks found in the same retention time frame. In both isopropanol and 2-butanol, L-serine was indistinguishable from the baseline noise. Chromatographic evidence of both

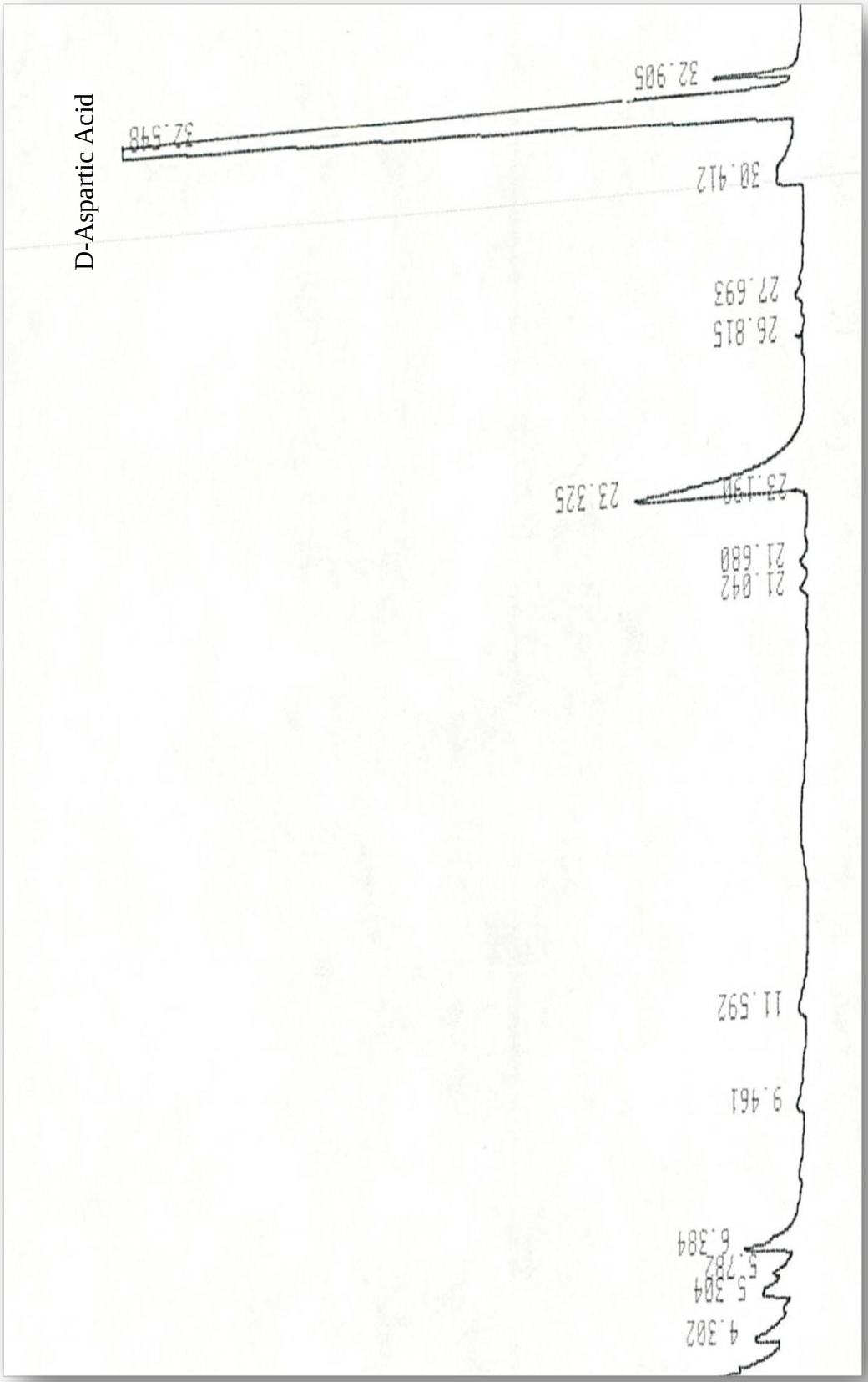


Figure 3: Chromatogram of D-aspartic acid with a retention time of 32.548 minutes.

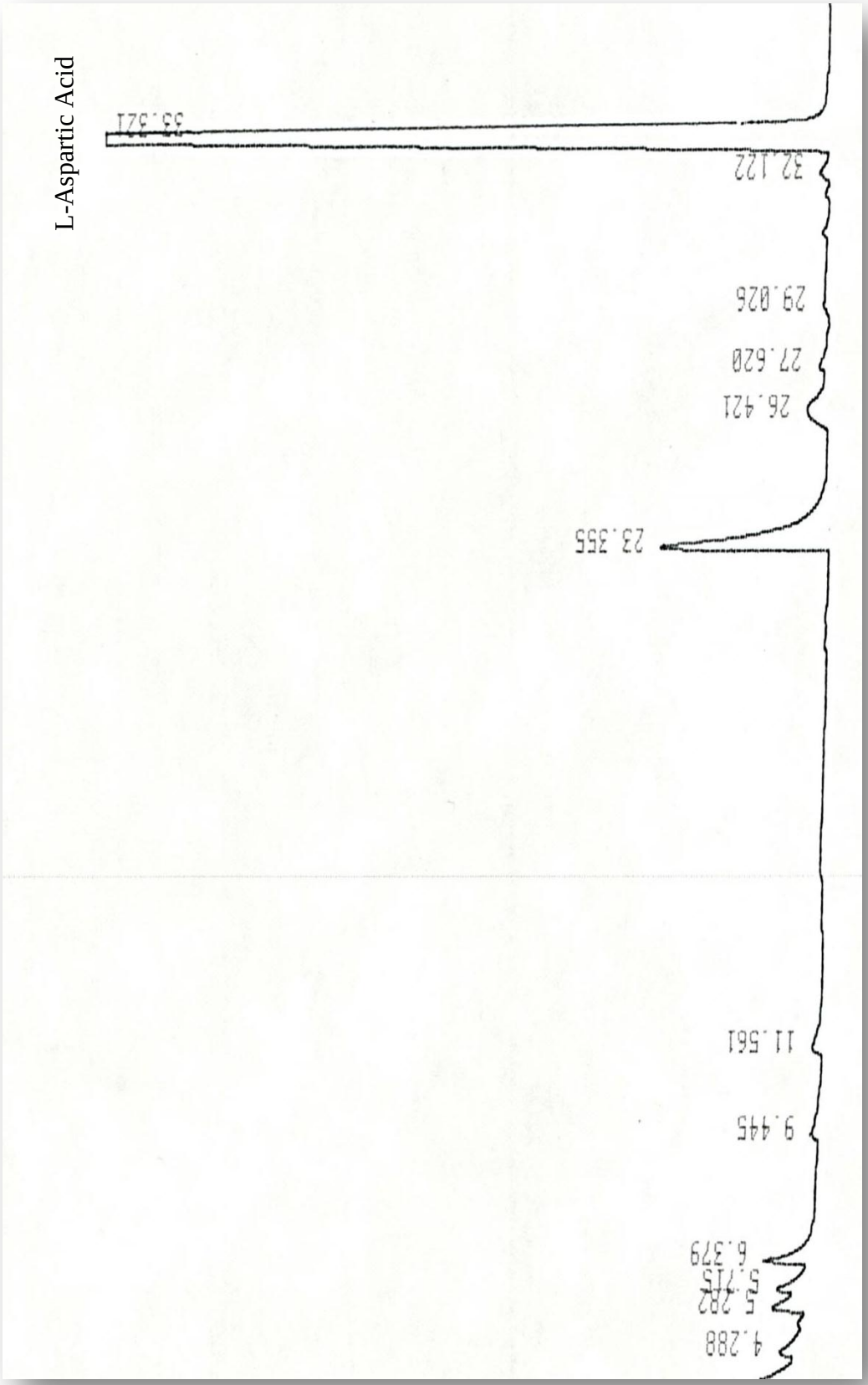


Figure 4: Chromatogram of L-aspartic acid with a retention time of 33.321 minutes.

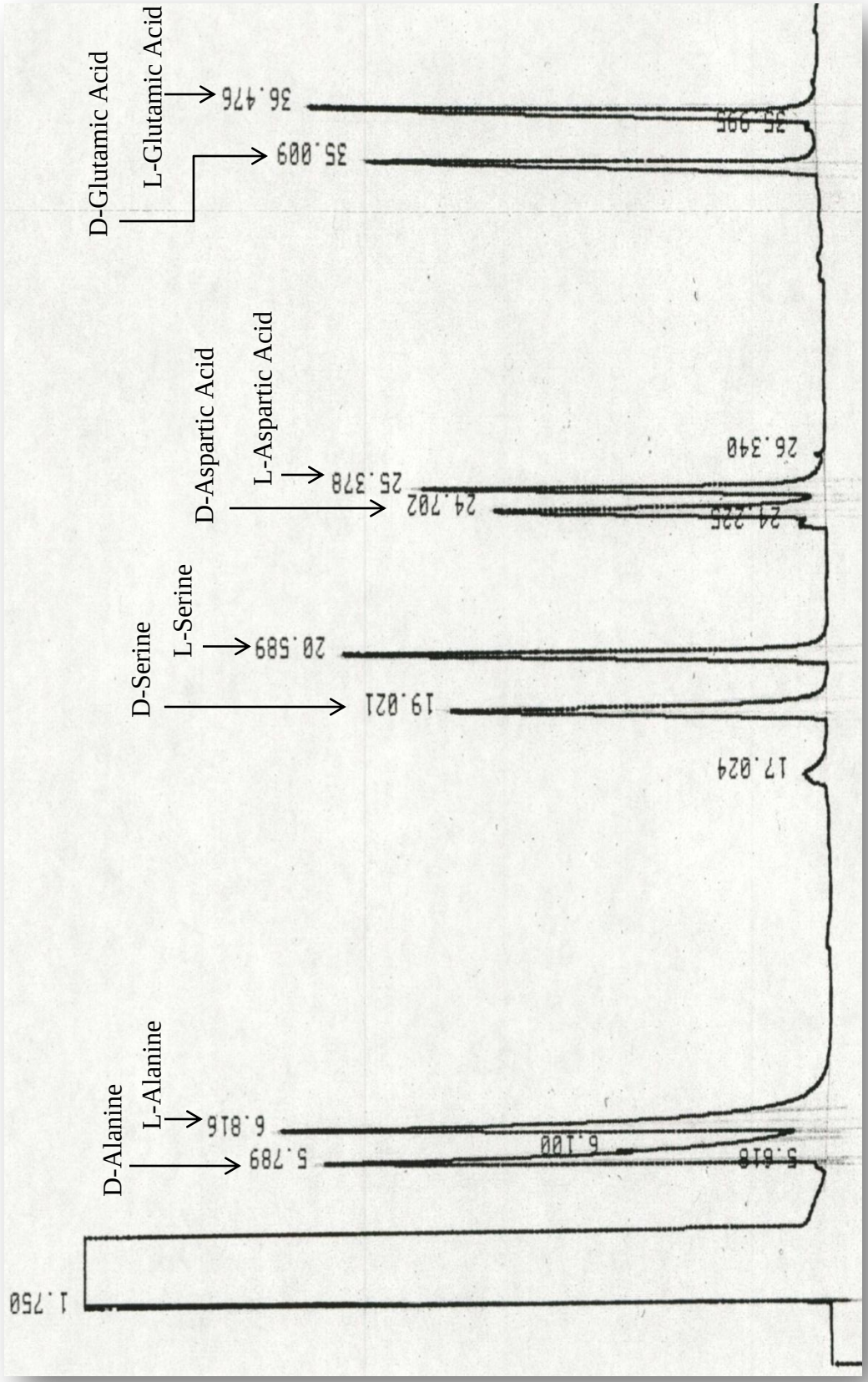


Figure 5: DL Standards derivatized using methanol as the esterification alcohol.

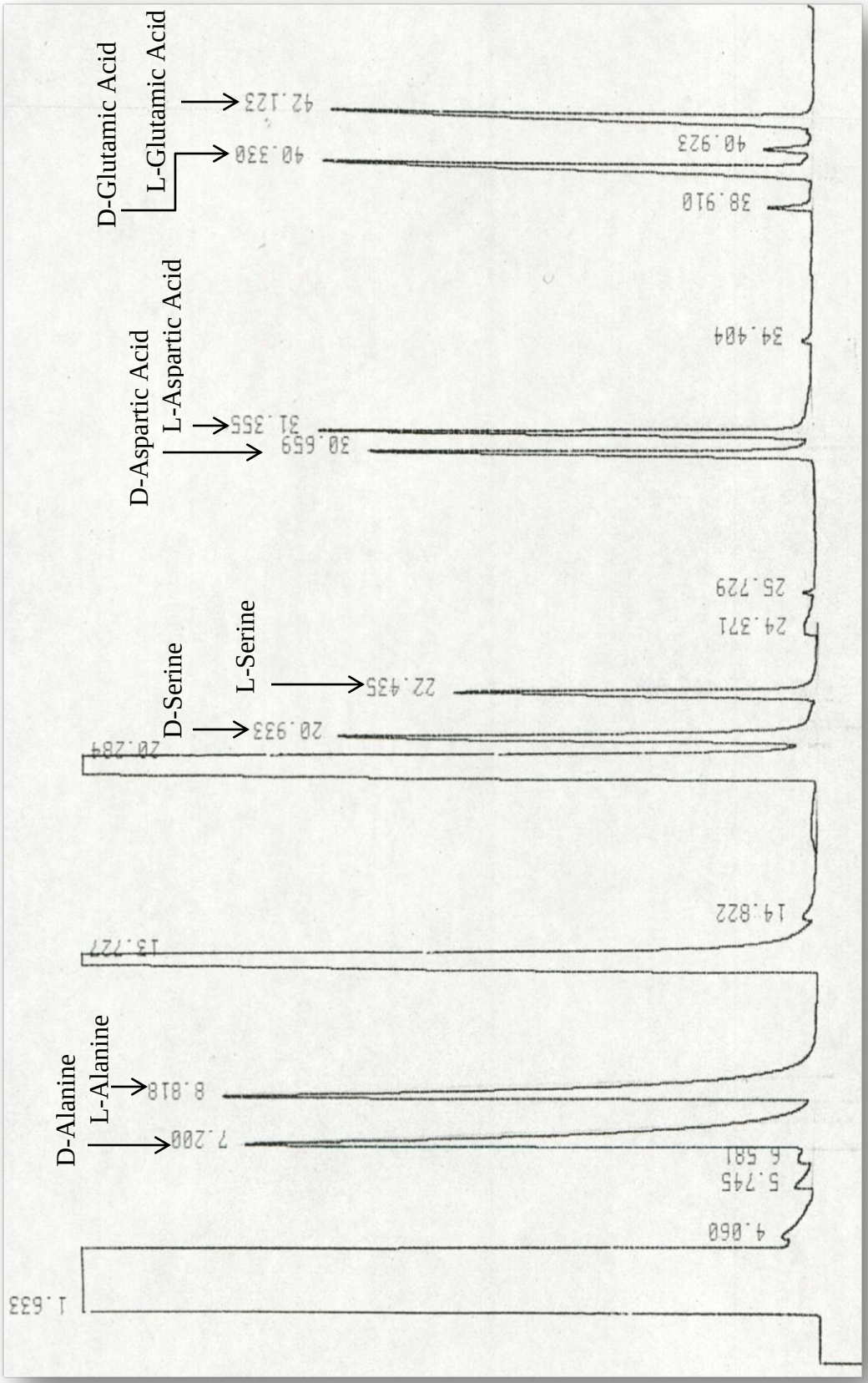


Figure 6: DL Standards derivatized using ethanol as the esterification alcohol.

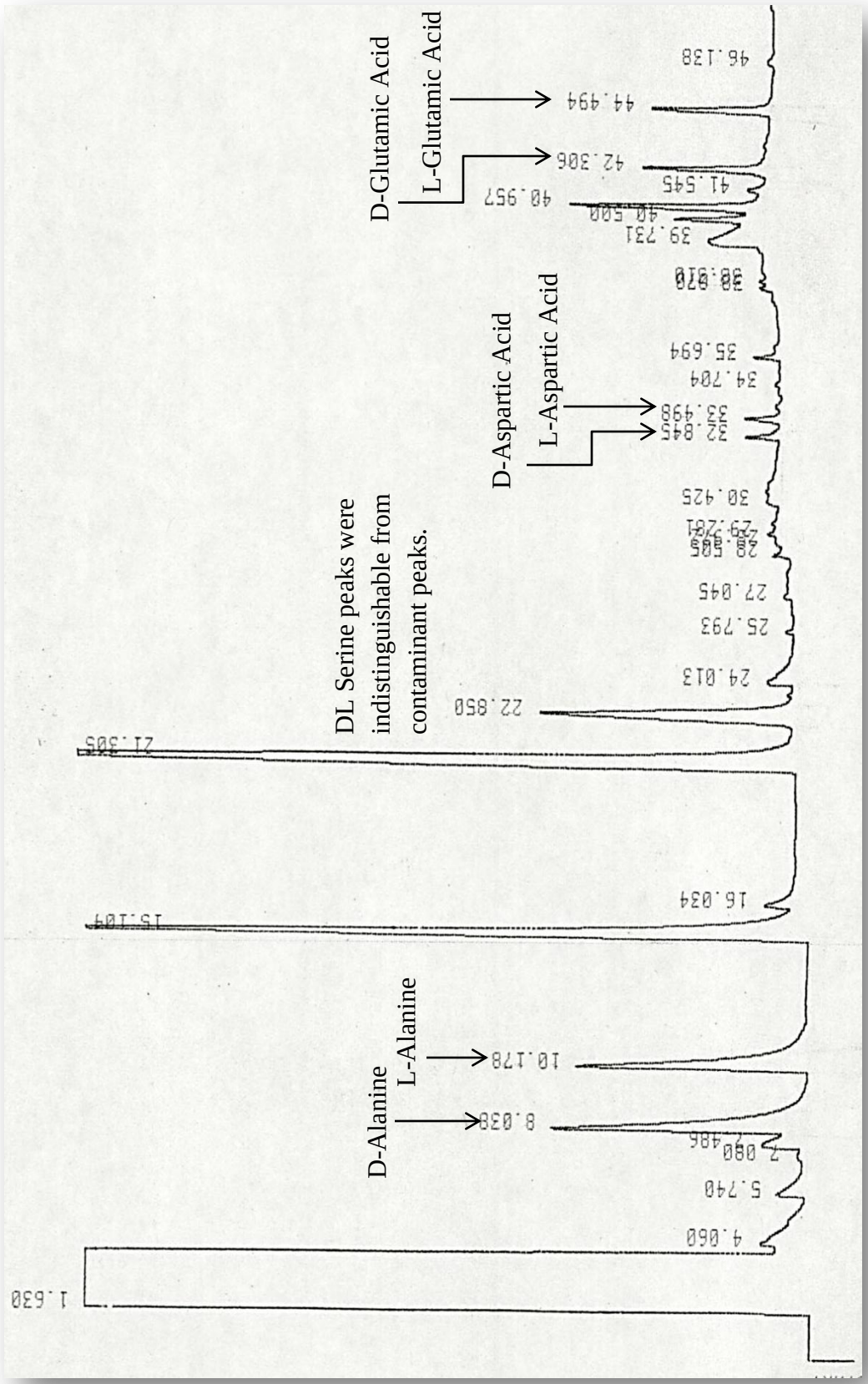


Figure 7: DL Standards derivatized using isopropanol as the esterification alcohol.

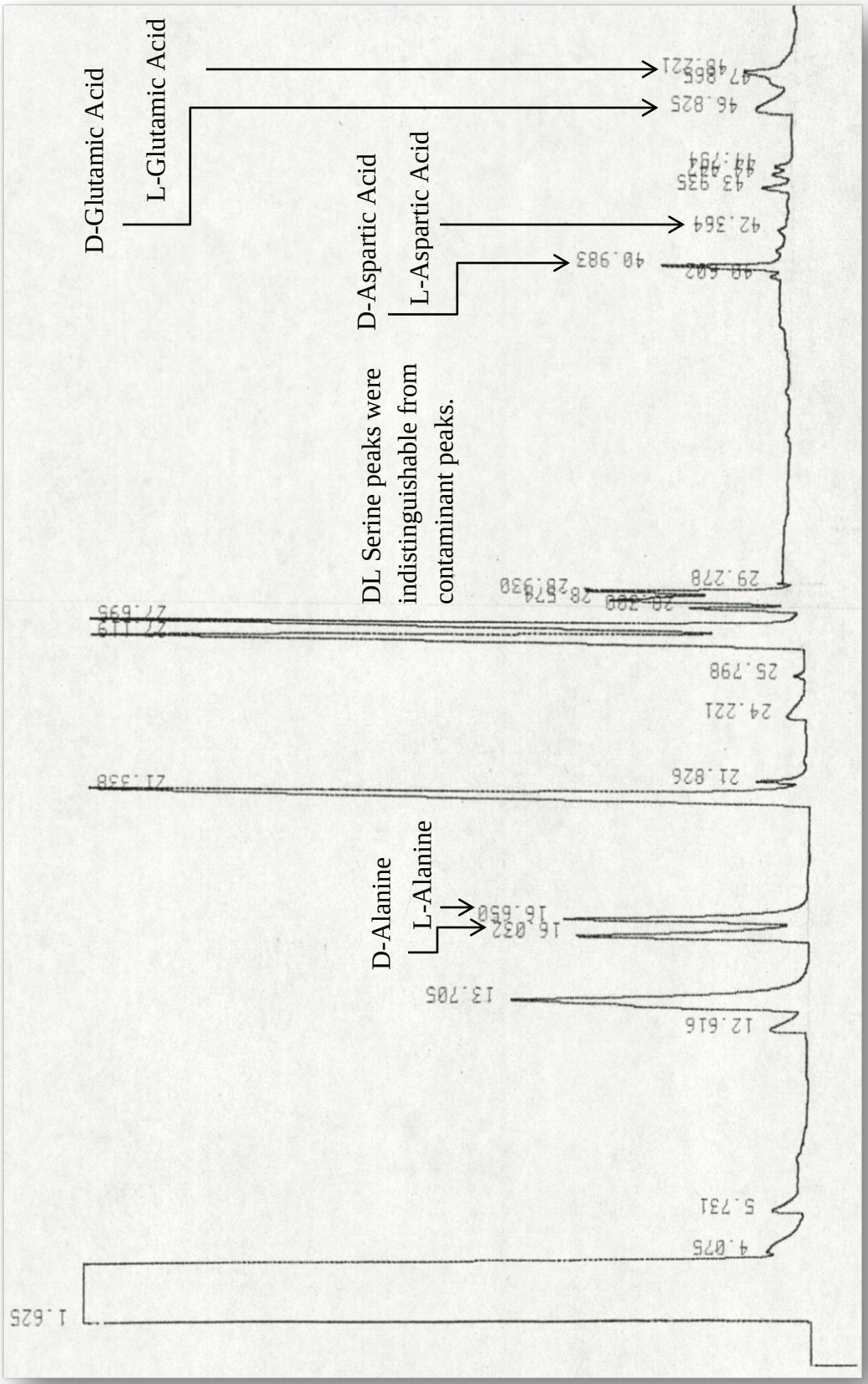


Figure 8: DL Standards derivatized using 2-butanol as the esterification alcohol.

isopropanol and 2-butanol appeared to have an increased number of peaks surrounding the amino acids of interest which could be attributed to contamination of non-derivatized elements. All amino acid peaks were distinguishable using methanol and ethanol.

D. Resolution of the Amino Acid Standard as a Function of Alcohol

Resolution was calculated of each amino acid for the four experimental esterification alcohols and graphed to show the efficiency (Figure 9). Methanol esterified amino acids demonstrated baseline resolution for all amino acid standards with the exception of alanine resolution which was 1.1. When ethanol was used as the esterification alcohol, baseline resolution was achieved for all amino acids present in the standards with the least resolved aspartic acid having a resolution of 1.5. Isopropanol again proved to be problematic in that only alanine and serine were distinguishable and therefore the only two amino acids available for resolution analysis at 2.5 and 2.0 respectively. In the case of 2-butanol, resolution for alanine at 1.1 fell below the convention of 1.2 for baseline resolution.

Due to the chromatographic issues discovered using isopropanol and 2-butanol, only methanol and ethanol remained of interest for selection as the esterification alcohol. When comparing the two chromatograms, ethanol resulted in slightly higher resolution than methanol with all peaks distinguishable for both. Based upon the chromatographic evidence, ethanol performed most advantageously for the amino acid standards and was chosen for this research.

Each alcohol was assessed for efficiency in resolution with the determination that ethanol performed most advantageously on the amino acid standards. This data was graphed to illustrate the effect of each alcohol on an individual standard amino acid

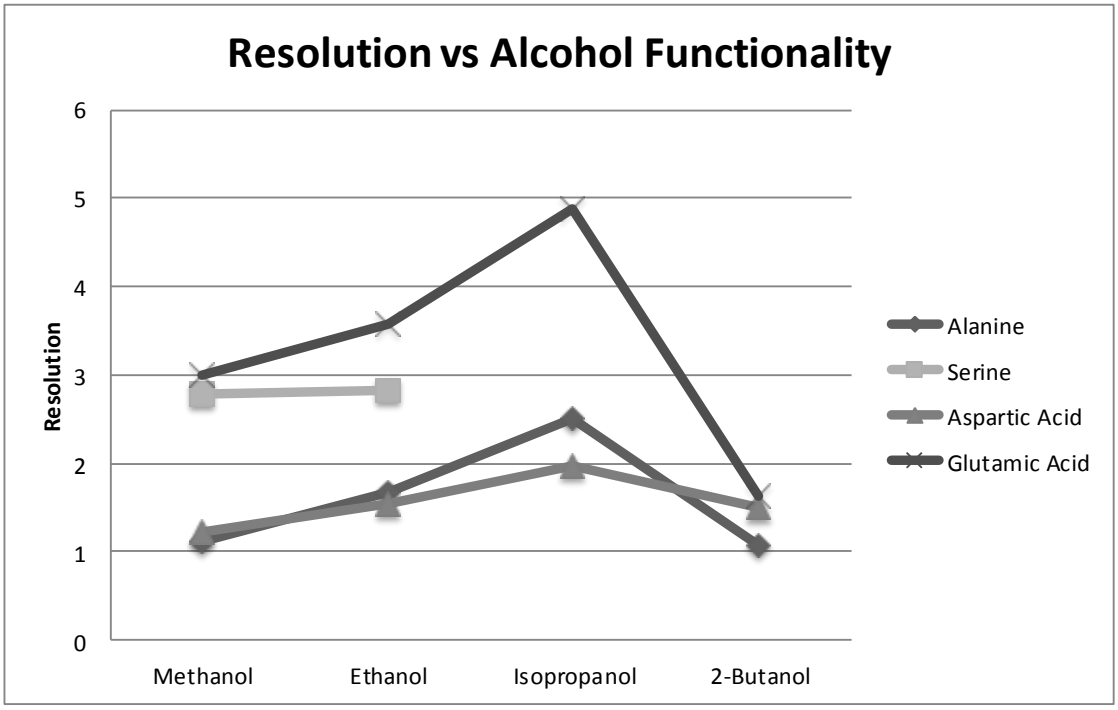


Figure 9: Graphical representation of the resolution of amino acid standards as a function of the alcohol used during the derivatization process.

(Figure 9). The figure clearly illustrates the dependency resolution has upon the alcohol used. Finally having determined an alcohol most suited for the standards, it is important to also test the utility when applied to a complex matrix such as dentin samples. Prior to performing the methodology used for the standards, acquisition of dentin is necessary.

E. Preparing the Tooth for Analysis

Prior to completing the esterification alcohol study on dentin, the tooth is prepared by cleaning followed by extraction. All teeth were donated from a local dentist who provided the age and tooth number following extraction from his patients. No information identifying the patient was used and therefore in compliance with the Health Insurance Portability and Accountability Act (HIPPA). The teeth removed from the dentist's patients were considered waste and therefore not subject to the Research on Human Subjects Policy. The teeth were categorized for research upon receipt of the researchers through an identification system involving the number of teeth from one person, the age at extraction, specific descriptors of the tooth such as number and type (i.e. Molar, Bicuspid, etc.), and by a chronologically assigned patient number based on the order in which it was received.

To prepare the tooth for dentin removal, any contaminants on the exterior of the tooth must be removed. The whole tooth was submerged in 0.2 M hydrochloric acid, followed by three successive water rinses, ethanol, and lastly ethyl acetate. The manner in which the washes were performed ranged from polar to non-polar. The hydrochloric acid removed polar materials while ethanol removed any intermediary polar materials, and lastly ethyl acetate removed any non-polar materials from the outer layer of the tooth. Once the tooth was dry, a series of cuts were made to remove the dentin.

Based upon previous research, a longitudinal cut was made from crown to root using a diamond blade affixed to a Dremel drill. Once the tooth had been cut in half (Figure 10) all material was cut away from the dentin. The dentin was then placed in a mortar and pestle and ground with as much efficiency as possible. However, this method yielded a large fraction of enamel contamination. The enamel is composed of a higher inorganic content than the organic proteinaceous material that is comprised of the amino acids. A Scanning Electron Microscopic image coupled with Energy Dispersive X-Ray analysis was implemented to evaluate the content differences among the layers of the tooth (Figure 11). The enamel layer resulted in high intensities of phosphorous and calcium content with a low carbon intensity. This is indicative of a higher percentage of inorganic material (P and Ca) (Figure 12) while the SEM-EDX analysis of the interior dentin layer showed a large increase in carbon intensity with a decrease in inorganic material (Tables 1 and 2). The amino acids are the building blocks of protein which are identifiable as organic material. Given this data, it is more prudent to extract primarily dentin with as little enamel contamination as possible. Cutting methodologies of past researchers yields a large contamination of inorganic material which can be readily viewed using a light microscope in which the snow-white crystalline structure of the enamel is visible. To avoid inclusion of the enamel, the method was modified to remove the top layer of the crown instead of a longitudinal cut which readily exposed the dentin layer below the surface (Figure 13). Once the crown was removed, a visible change in composition can be seen prominently under a light microscope (Figure 14). A visible line of demarcation between the enamel layer and the internal dentin layer was visible as

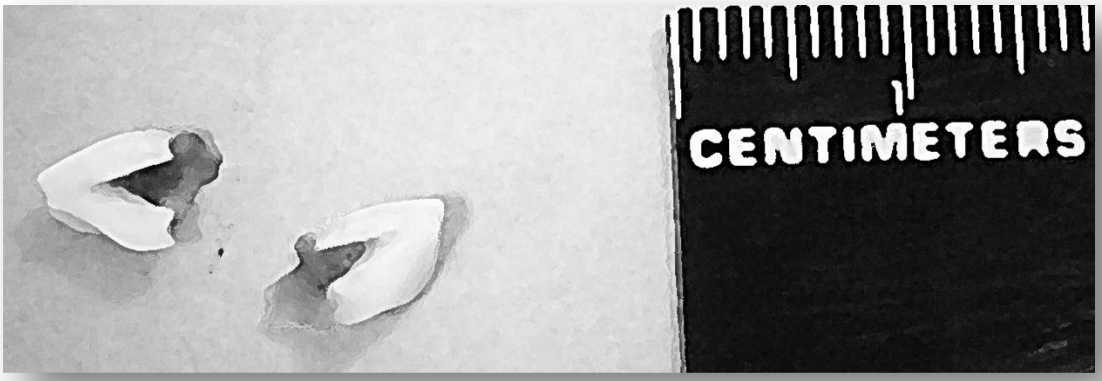


Figure 10: Longitudinal cut made from the crown to the root exposing the dentin from the initial cutting procedure.

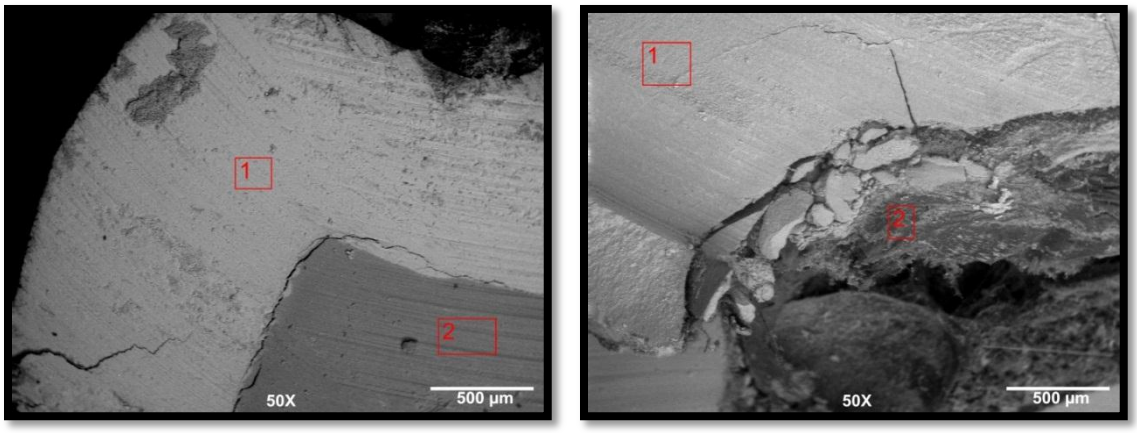


Figure 11: (Left) JEOL JSM-5400 LV SEM-EDX images of enamel (light) to dentin (dark). (Right) JEOL JSM-5400 LV SEM-EDX image of dentin (light) to pulp (dark).

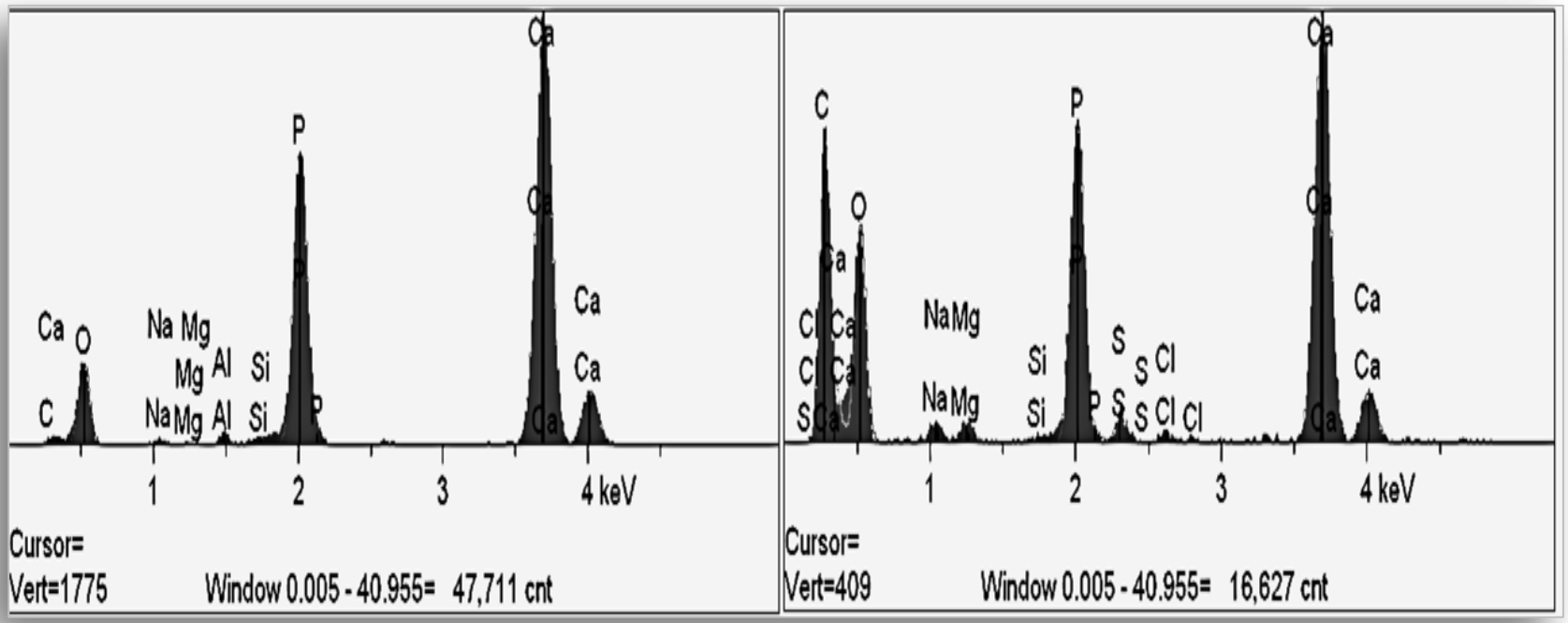


Figure 12: (Left) SEM-EDX enamel composition taken of the interior of the tooth. (Right) SEM-EDX dentin composition taken of the interior of the tooth.

Table 1

**SEM-EDX results of the internal analysis of the enamel in a tooth sample**

| Element | Line | Intensity (c/s) | Error 2-sig | Atomic % | Concentration | Units | |
|---|---|---|---|---|---|---|---|
| C | Ka | 0.74 | 0.488 | 1.28 | 0.68 | wt.% | |
| O | Ka | 55.06 | 1.976 | 65.18 | 46.27 | wt.% | |
| Na | Ka | 3.83 | 0.997 | 0.70 | 0.72 | wt.% | |
| Mg | Ka | 1.39 | 1.063 | 0.16 | 0.17 | wt.% | |
| Al | Ka | 6.37 | 1.377 | 0.51 | 0.61 | wt.% | |
| Si | Ka | 8.91 | 1.455 | 0.56 | 0.70 | wt.% | |
| P | Ka | 235.49 | 4.145 | 13.27 | 18.24 | wt.% | |
| Ca | Ka | 381.77 | 5.160 | 18.33 | 32.60 | wt.% | |
| | | | | 100.00 | 100.00 | wt.% | Total |

Table 2

**SEM-EDX results of the internal analysis of the dentin in a tooth sample**

| Element | Line | Intensity (c/s) | Error 2-sig | Atomic % | Concentration | Units | |
|---|---|---|---|---|---|---|---|
| C | Ka | 37.72 | 1.622 | 46.25 | 34.43 | wt.% | |
| O | Ka | 31.95 | 1.498 | 43.05 | 42.69 | wt.% | |
| Na | Ka | 3.30 | 0.766 | 0.71 | 1.01 | wt.% | |
| Mg | Ka | 3.58 | 0.854 | 0.47 | 0.71 | wt.% | |
| Si | Ka | 1.67 | 0.920 | 0.13 | 0.22 | wt.% | |
| P | Ka | 57.86 | 2.161 | 3.93 | 7.54 | wt.% | |
| S | Ka | 4.37 | 1.024 | 0.29 | 0.58 | wt.% | |
| Cl | Ka | 1.84 | 0.862 | 0.11 | 0.25 | wt.% | |
| Ca | Ka | 87.89 | 2.532 | 5.06 | 12.57 | wt.% | |
| | | | | 100.00 | 100.00 | wt.% | Total |

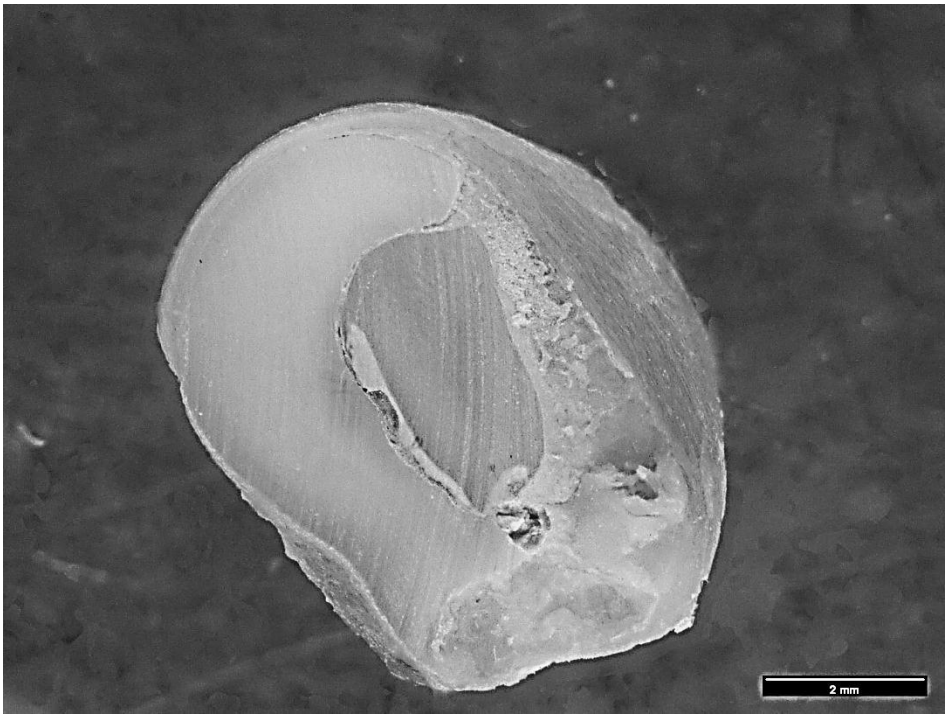


Figure 13: Overhead view of the crown removal to expose the dentin layer below.

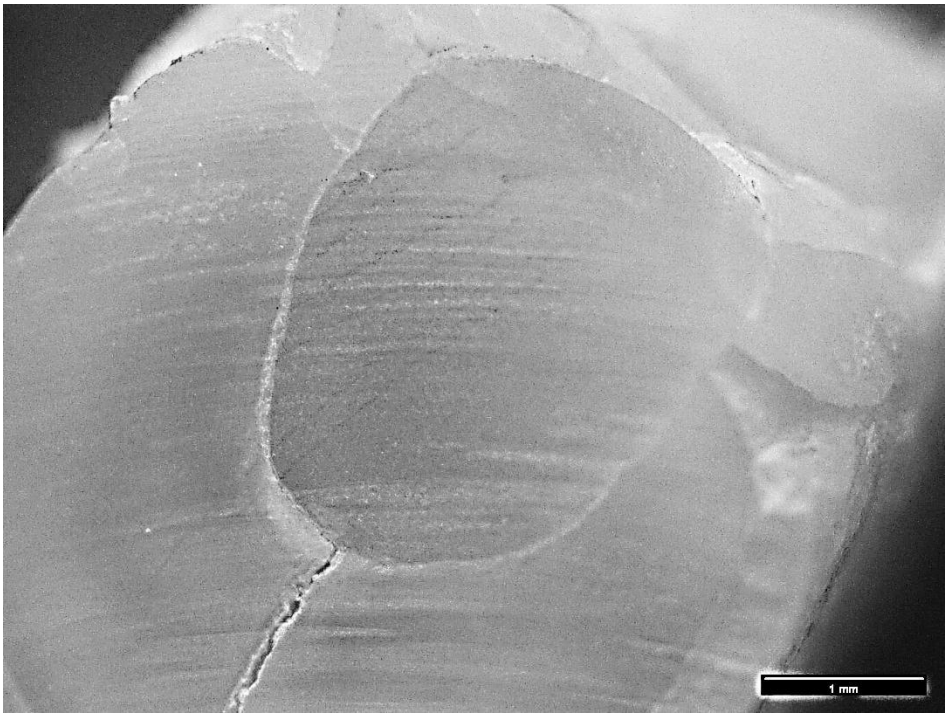


Figure 14: Prominent line of demarcation between enamel and dentin material.

well as a marked color change from a white-yellow to a darker yellow-light orange. This outlined area allowed for a more controlled dentin extraction to be made using a diamond tip drill bit due to the visibly distinctive layers (Figure 15). The drill bit fashioned the dentin into a fine powder which is more preferred than that offered by the mortar and pestle method, which yields a larger granular product (Figure 16). The isolated dentin was viewed by microscopy and yielded a more visible yellow appearance and smaller crystalline powder. The smaller particle size increases the surface area for reaction which in turn yields a higher conversion and hence recovery during the isolation of the amino acid. The increased yield affords a more accurate representation of the amino acid content in dentin. It was found during this study that molars are most advantageous to analyze due to size and therefore larger dentin content. Excision from the molars produced higher yields of dentin due to the size of the tooth and a readily apparent demarcation between enamel and dentin. Larger teeth such as molars were found to have a larger internal diameter of dentin that the canines and bicuspids. Therefore, for this study, molars were preferable but not always an option. Care was taken when excising dentin from teeth with smaller diameters but often yielded approximately 20-30 mg of material as compared to 60-80 mg from molars. This could also be problematic due to the inclusion of enamel in the collected sampled diluting out the proteinaceous material.

F. Isolation of the Amino Acids from the Dentin Matrix

The matrix of the dentin is complex and the amino acids must be isolated to avoid any chromatographic overlap from non-amino acid materials. To accomplish this, a cation exchange column was utilized containing Dowex® 50W-X8, 50-100 mesh. The Dowex cation exchange column contains a styrene-divinylbenzene matrix with an acidic

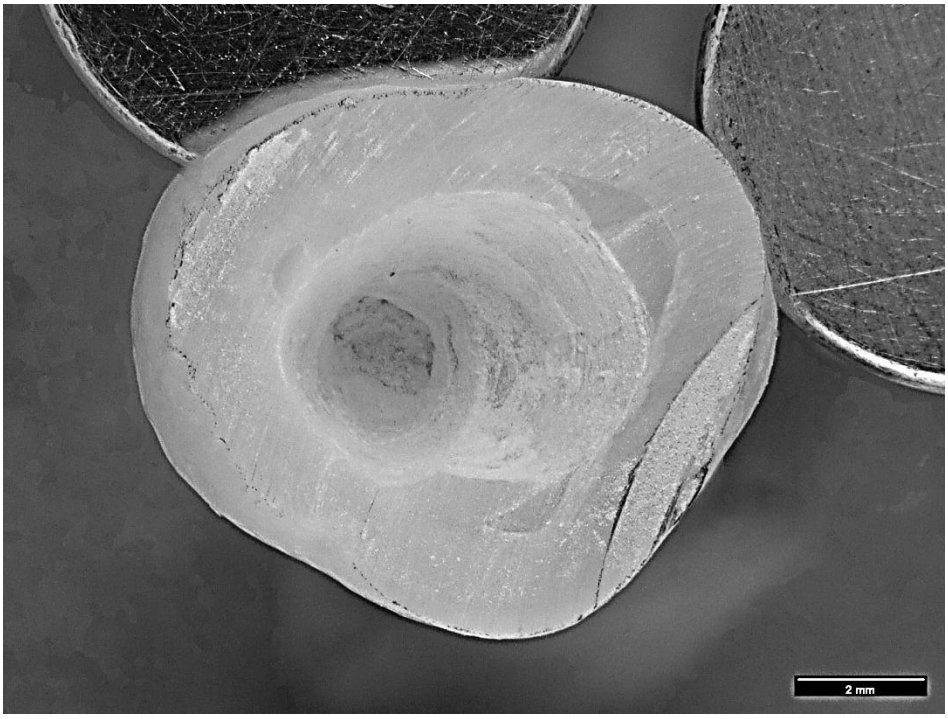


Figure 15: Internal view of the tooth post-dentin extraction using a diamond tip drill bit.


Figure 16: Light microscope image of the resulting fine powdered dentin after extraction using a diamond tip drill bit.

sulfonic acid functional group. The copolymer ST-DVB creates the stationary phase matrix while the sulfonic acid functional group alters the hydrophobicity and thus changes the retention on the column. This allows for effective isolation and separation of amino acids for analysis. Prior to placement on the column, the dentin sample undergoes a 6 hour hydrolysis with 6 M hydrochloric acid which liberates and acidifies amino acids. Once applied to the acidic column, ion exchange takes place and retains the acidified amino acids allowing the bulk of the remaining matrix to elute from the column. Previous research found efficient isolation of the amino acids using these specifications for the ion exchange column. To elute the retained amino acids, 2.0 M aqueous ammonia was used.

To determine the optimum amount of resin and elution volume, a series of resin volumes were investigated to ensure the highest recovery. In order to accomplish this task, an amino acid mixture containing D/L alanine, serine, aspartic acid, and glutamic acid standards was utilized. The resin volume was modified for four separate elutions to determine optimum recovery. A control was prepared to serve as the basis of percent recovery. For this purpose, a mixed standard was derivatized without being placed on the cation exchange column. Four remaining samples were placed individually onto the column that varied from approximately 1 cm, 2 cm, 3 cm, and 4 cm respectively. Data collected from the study illustrated the highest recovery occurred using 1 cm of resin volume (Figures 17 and 18). However, when looking at the chromatographic evidence, the 1 cm resin allowed for contamination that was not properly removed during cation exchange (Figure 19). Between 2 and 3 cm (Figures 20 and 21), recovery was still high

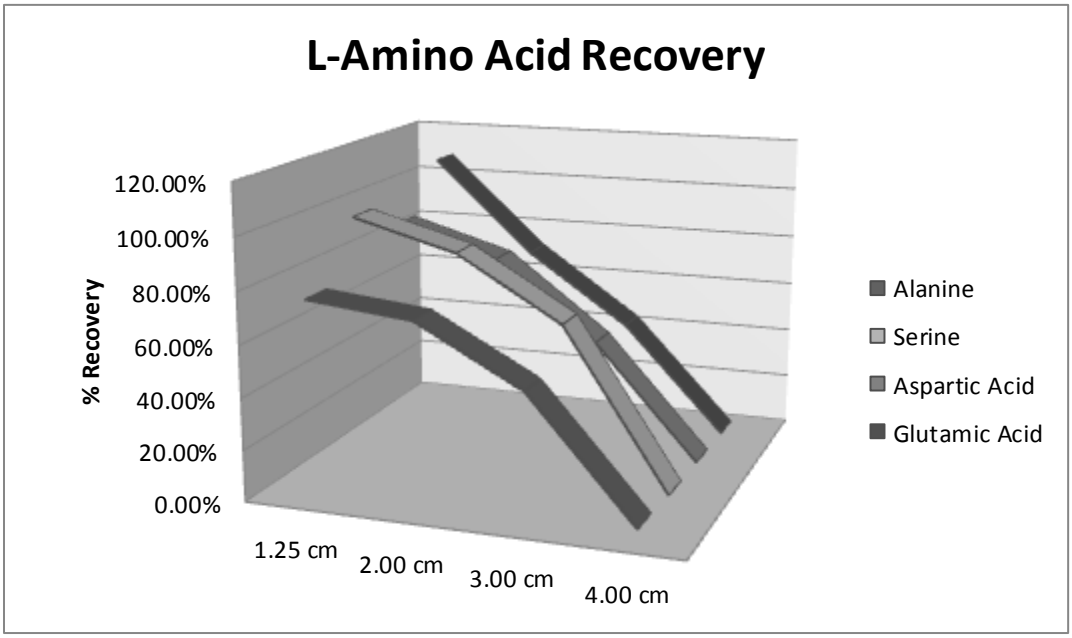


Figure 17: Percent recovery of L-amino acids as a function of resin column volume.

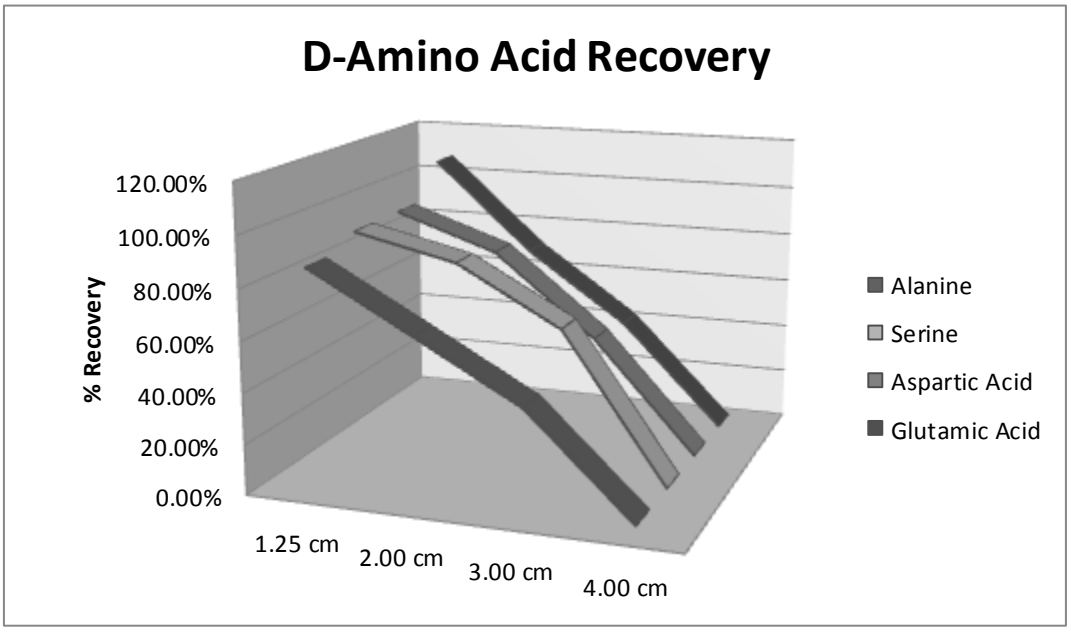


Figure 18: Percent recovery of D-amino acids as a function of resin column volume.

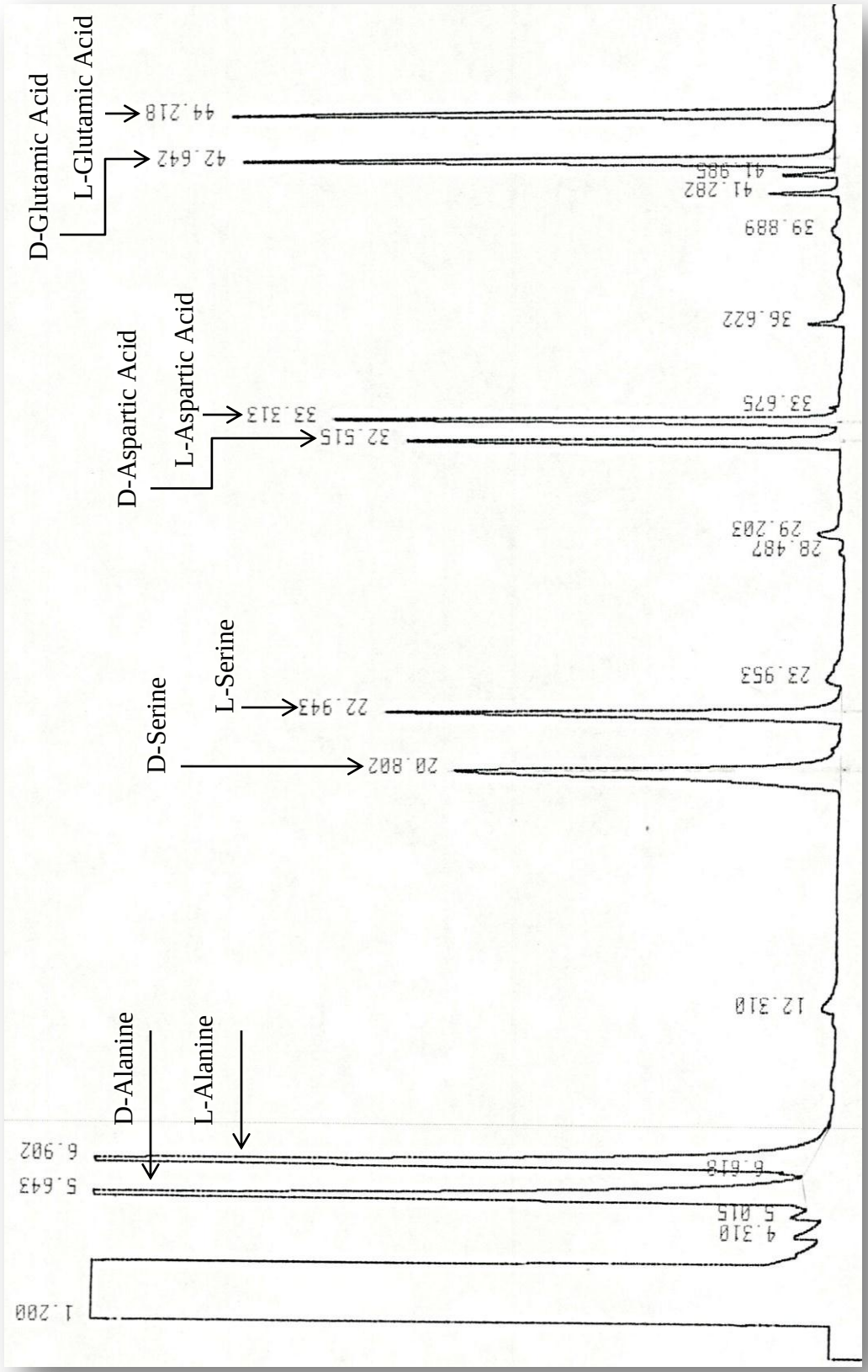


Figure 19: DL Standard isolated using 1.25 cm volume cation exchange resin.

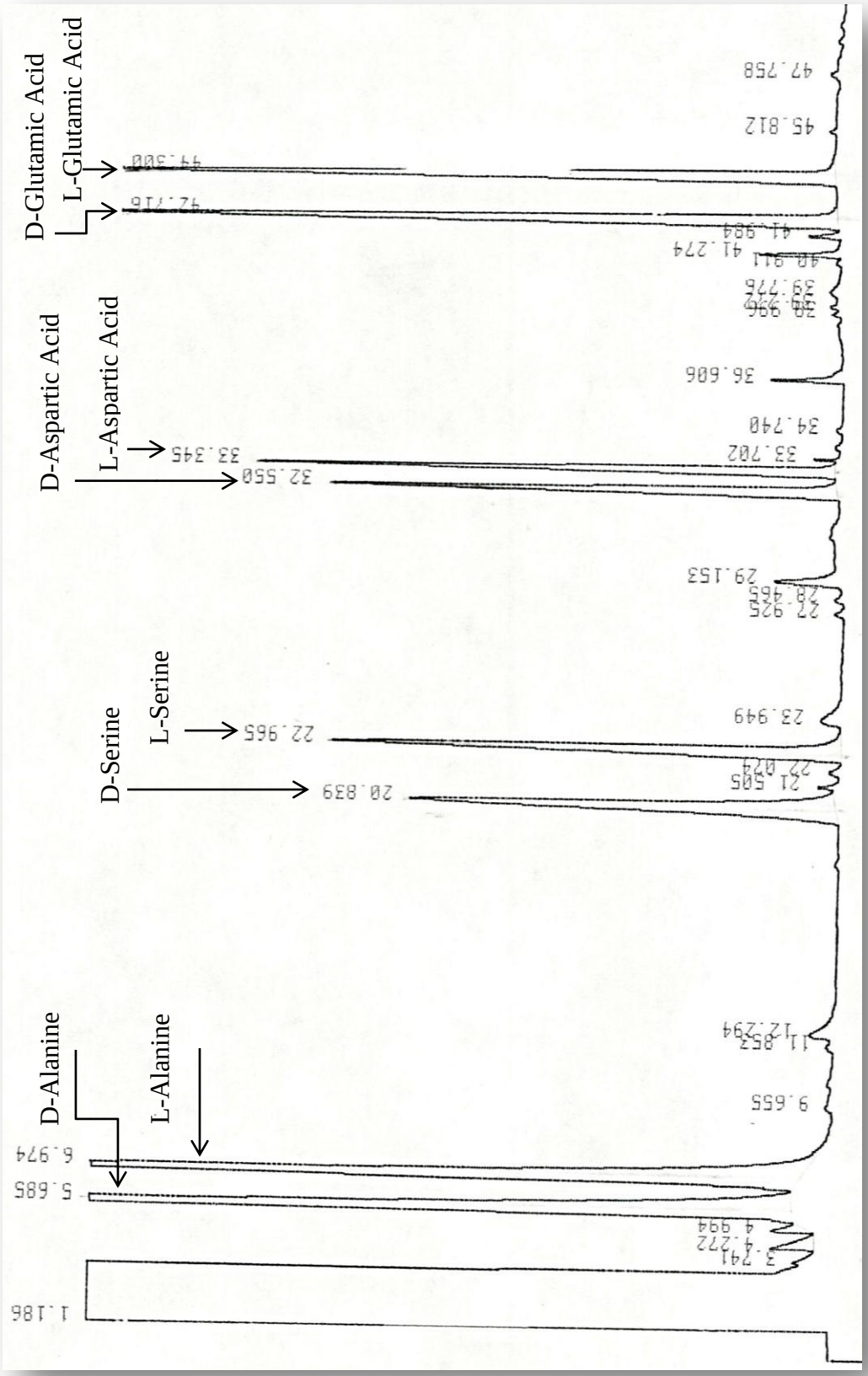


Figure 20: DL Standard isolated using 2.00 cm volume cation exchange resin.

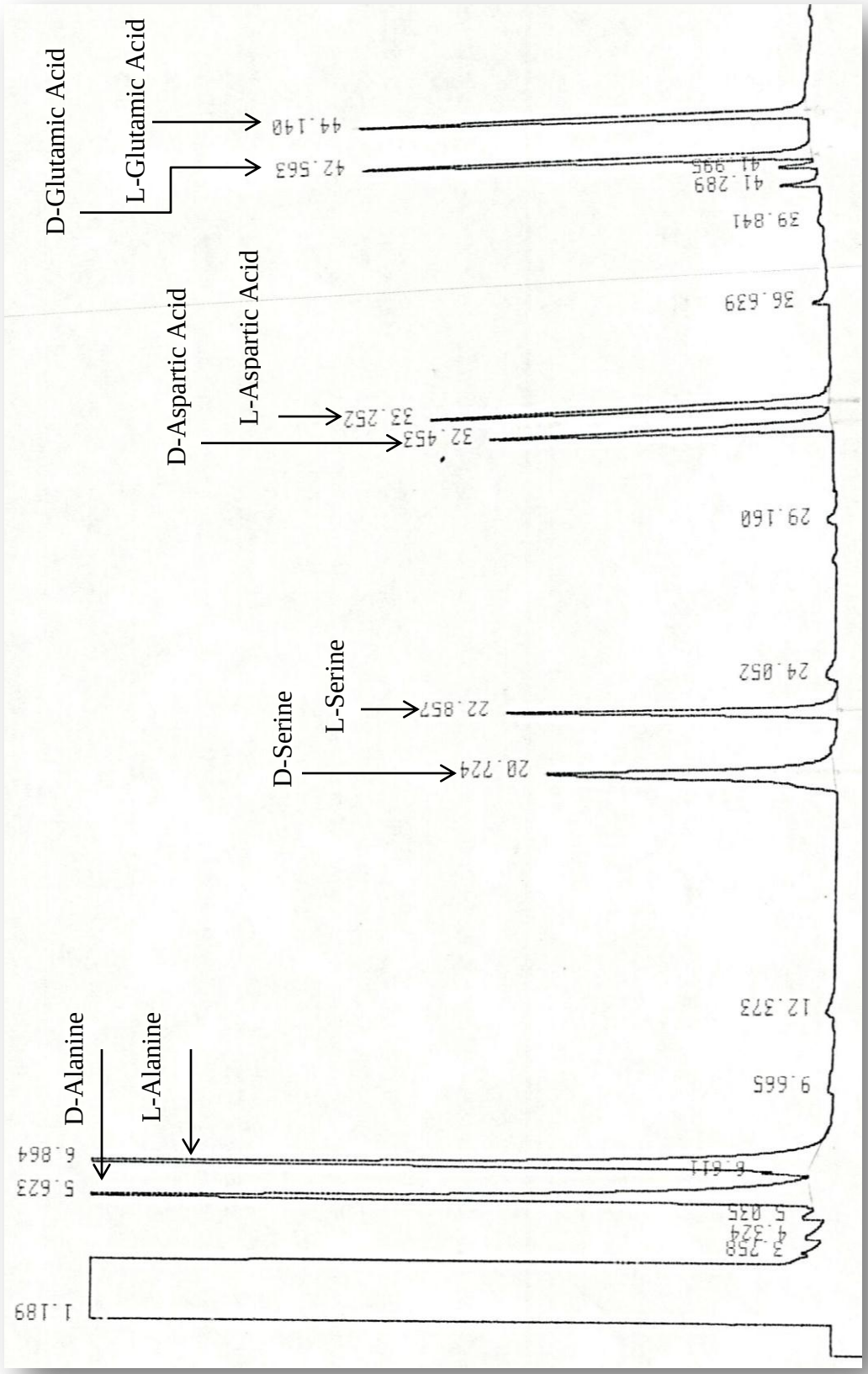


Figure 21: DL Standard isolated using 3.00 cm volume cation exchange resin.

and the chromatograph appeared much less contaminated while almost no recovery was found using 4 cm (Figure 22) of resin volume. Based upon the resin volume study, it was determined the amino acids should be isolated with a cation exchange resin volume of approximately 2.5 cm. Percent recovery was calculated for each amino acid in which the D-amino acid peak heights were individually calculated against its like amino acid height as was each of the L-conformations (Figure 23).

G. Alcohol Dentin Analysis

Methodologies established in the previous two sections prepared the dentin sample for the initial esterification step to analyze resolution as a function of the alcohols. As with the alcohol standards, an esterification alcohol will be determined based upon the presence or absence of the peaks as well as the resolution.

H. Distinguishing the Peaks

Chromatograms for methanol and ethanol held no complications in the distinguishability of the amino acid peaks (Figures 24 and 25). Like with the standards, isopropanol and 2-butanol showed deficiencies in the ability to distinguish the amino acid peaks (Figures 26 and 27). Serine and glutamic acid were distinguishable at this juncture but aspartic acid was undetectable in both chromatograms for isopropanol and 2-butanol. There is a noticeable drift in the baseline for isopropanol accompanied by a large bell-shaped peak which may hold aspartic acid but is completely indistinguishable from not only D- and L- conformations but any possible peak in the area.

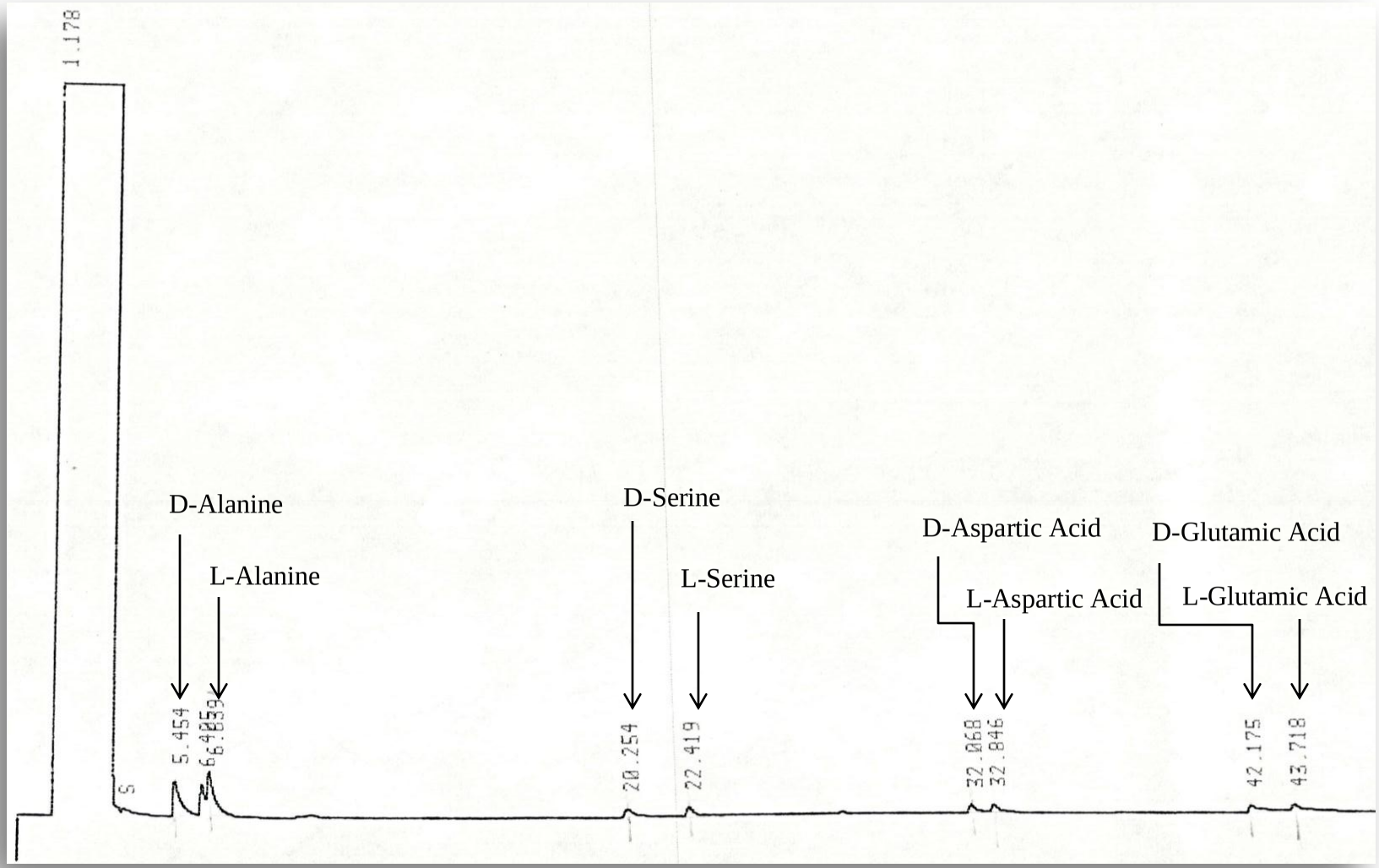


Figure 22: DL Standard isolated using 4.00 cm volume cation exchange resin.

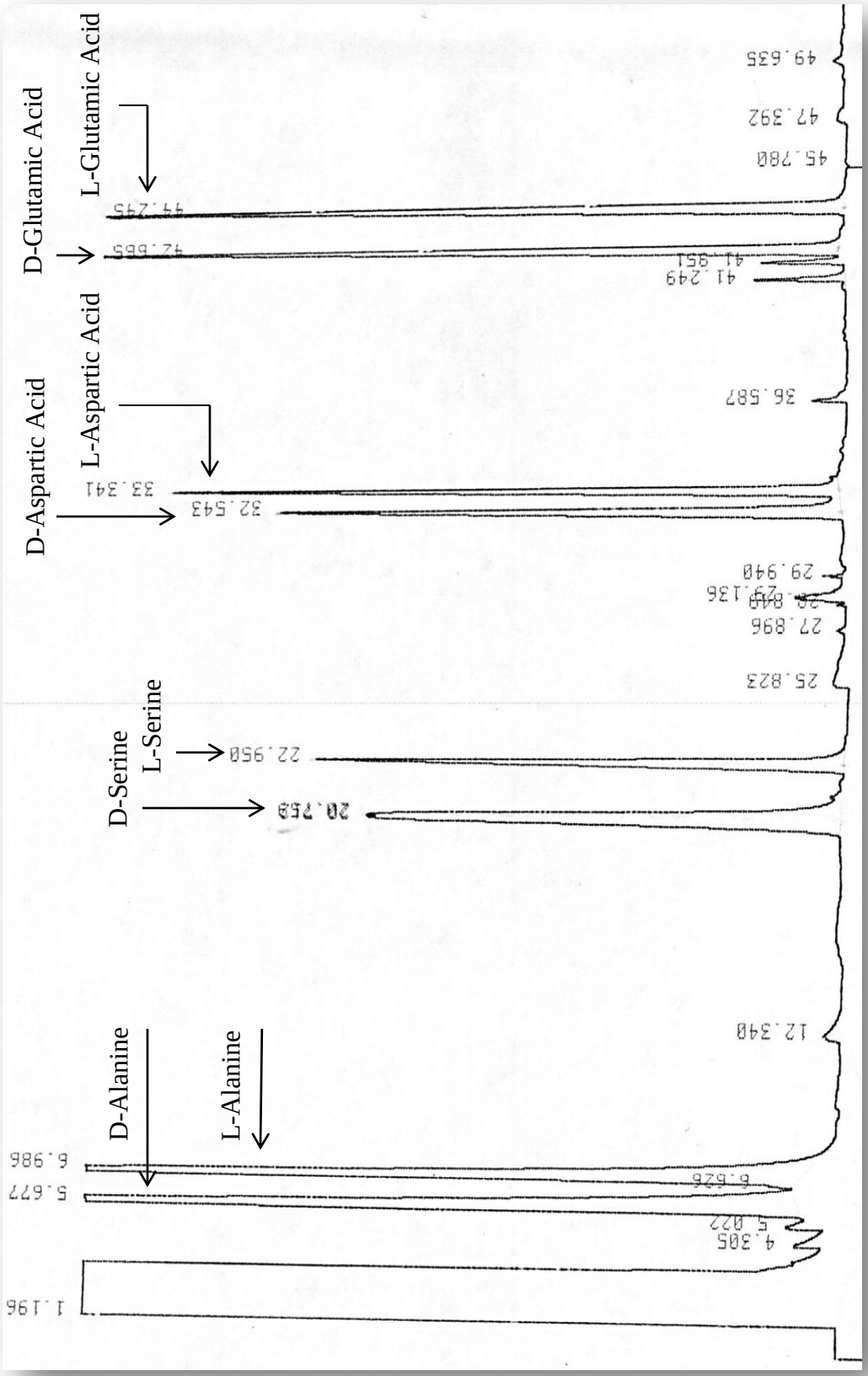


Figure 23: DL Standard receiving no treatment on the resin column and used for percent recovery calculations as the control.

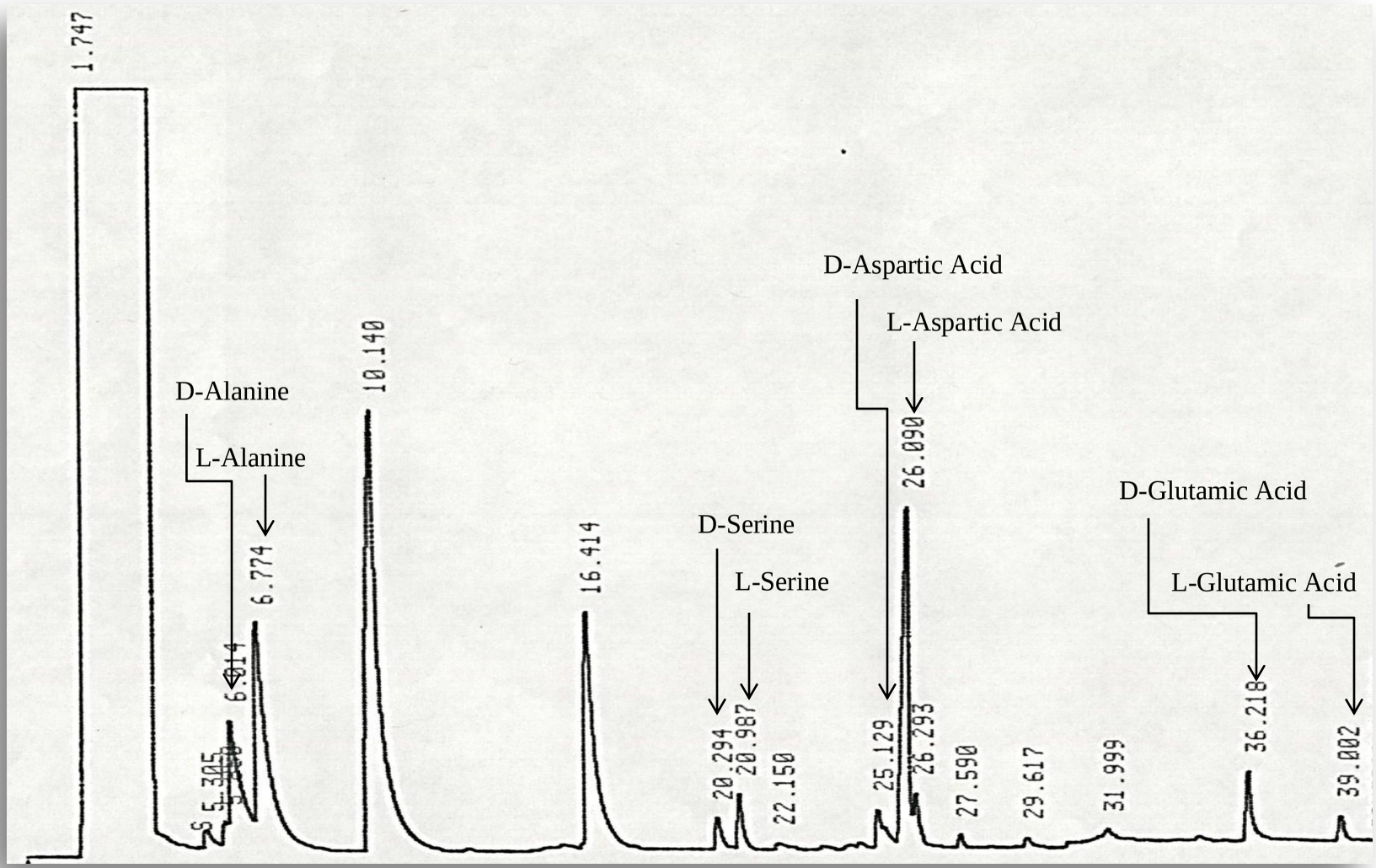


Figure 24: Chromatogram of derivatized tooth dentin utilizing methanol as the esterification alcohol.

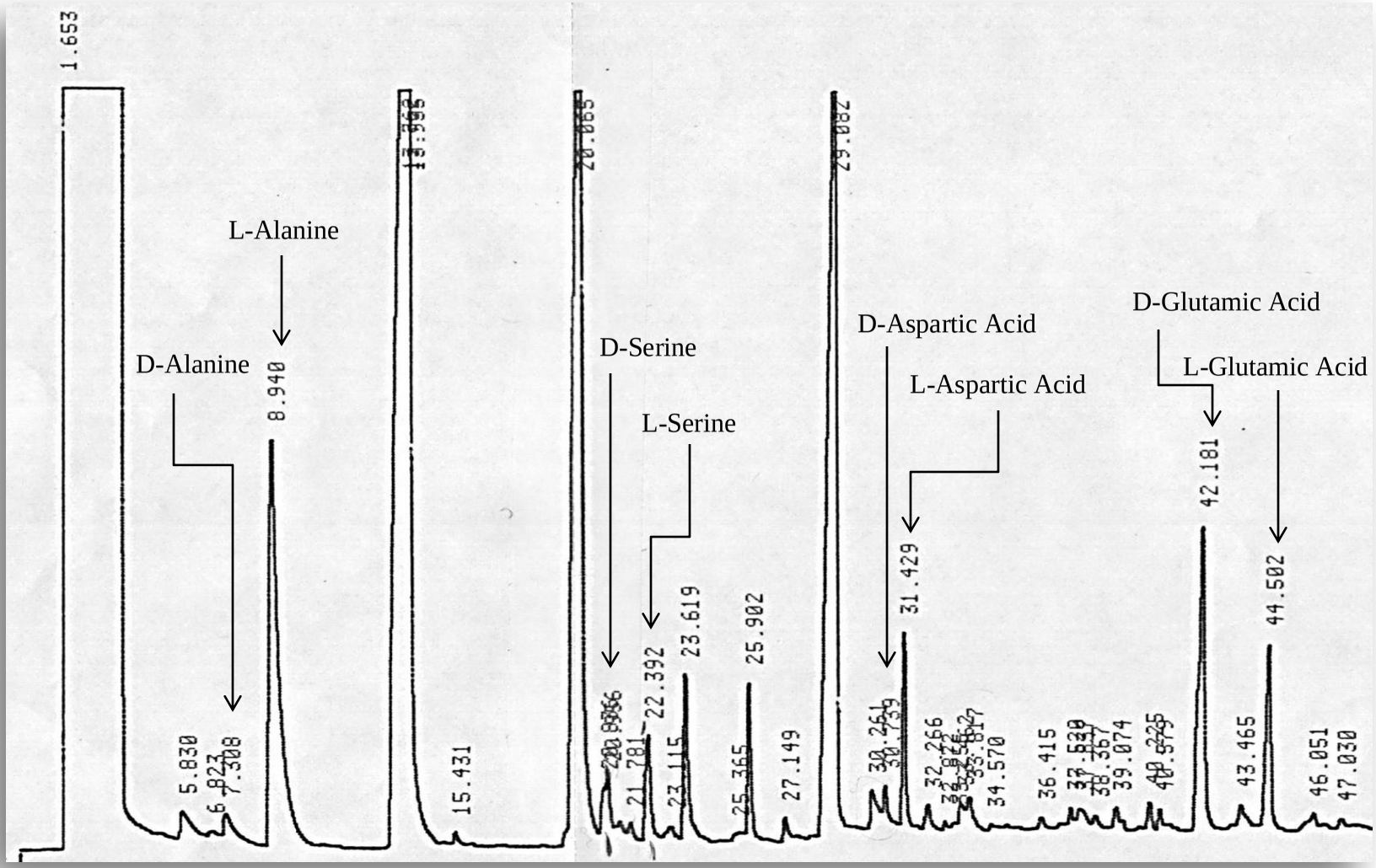


Figure 25: Chromatogram of derivatized tooth dentin utilizing ethanol as the esterification alcohol.

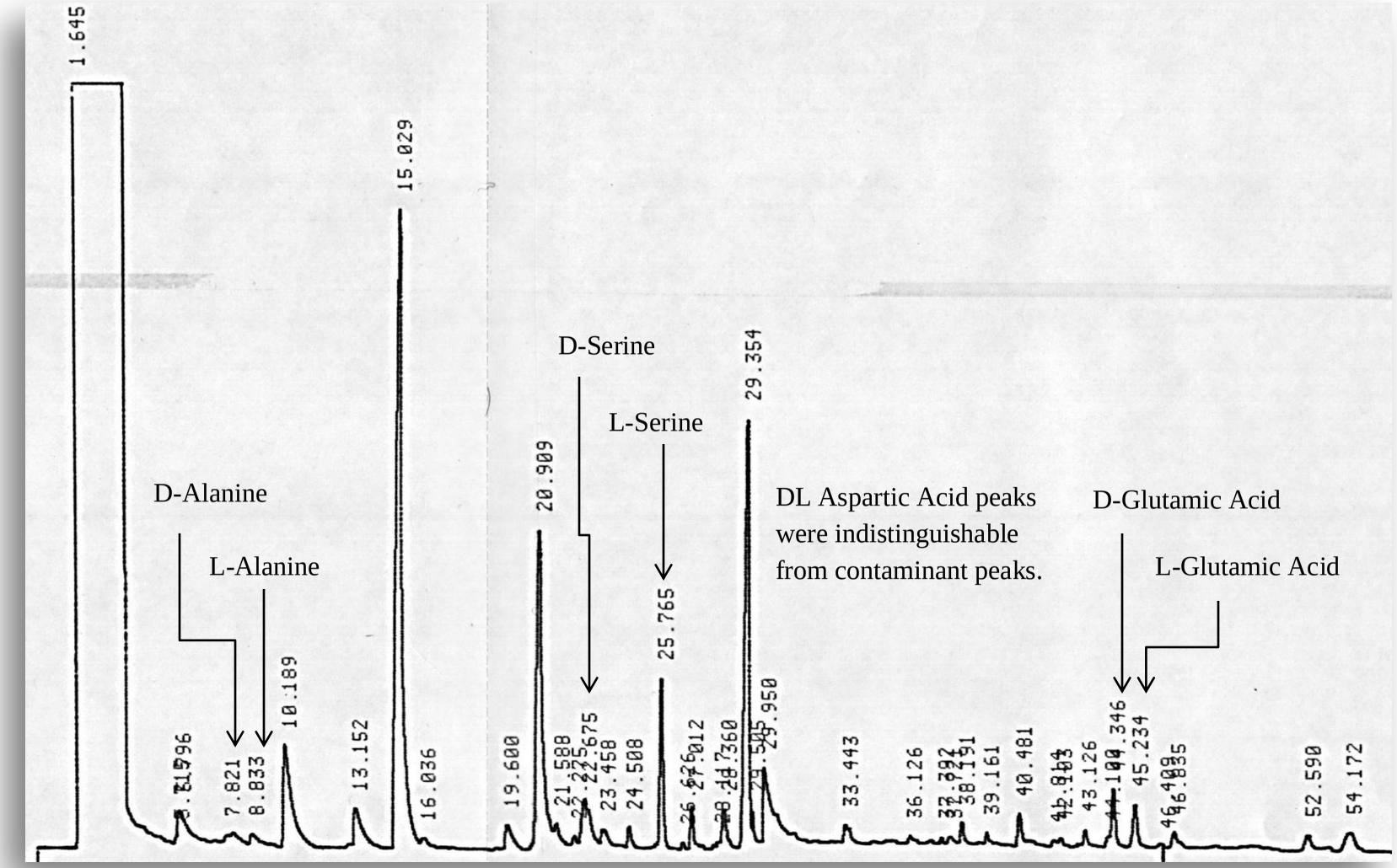


Figure 26: Chromatogram of derivatized tooth dentin utilizing isopropanol as the esterification alcohol.

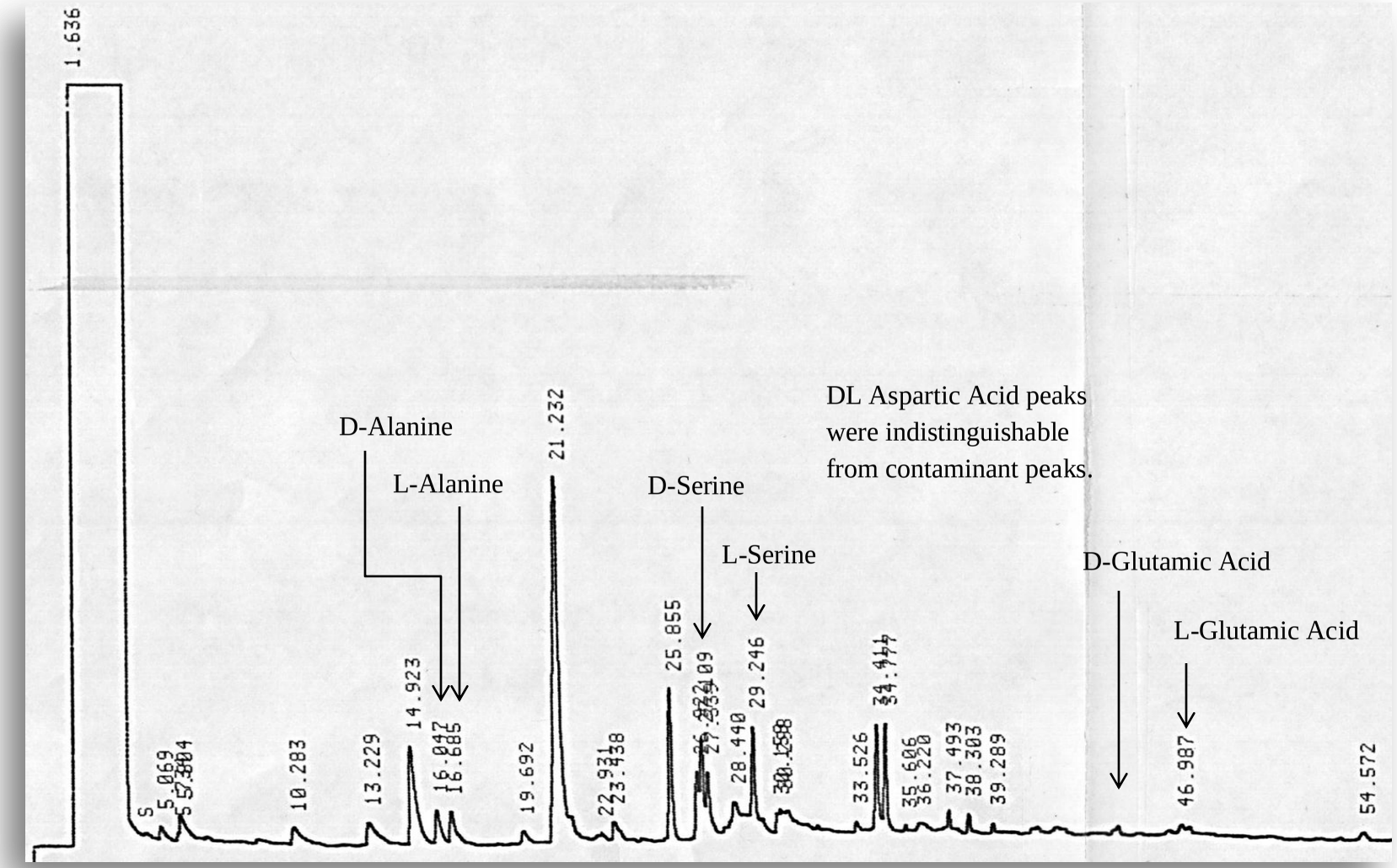


Figure 27: Chromatogram of derivatized tooth dentin utilizing 2-butanol as the esterification alcohol.

I. Resolution of the Dentin Sample as a Function of Alcohol

Resolution for each amino acid was calculated and was graphed to illustrate the effect of alcohol on dentin (Figure 28). Methanol treated dentin samples provided resolutions of 2.2 and 3.0 for serine and glutamic acid respectively. However, alanine and aspartic acid were unsuccessfully baseline resolved at 0.9 and 1.1 respectively. Ethanol proved to elicit baseline resolution with the least resolved amino acids being alanine and aspartic acid at 2.0. A dramatic decrease occurred in resolution using isopropanol and 2-butanol in comparison to ethanol. Resolution for alanine using isopropanol fell to 1.0 which is insufficient for baseline resolution. It is important to note that aspartic acid was undetectable using isopropanol and 2-butanol. In the case of 2-butanol, resolution for glutamic acid rose to 7.2 but fell below the conventional standard for both alanine and serine at 0.7 and 1.0 respectively. In agreement with the alcohol chosen from the amino acid study, ethanol as the esterification alcohol produced distinguishable peaks and resolution for all amino acids and was therefore chosen as the most suitable for this research.

J. Alcohol Size Affects Esterification

Amino acids range in size from small and sterically available to large and sterically hindered (Figure 29). They are very polar making it necessary to derivatize them before analysis by gas chromatography. Alanine being the smallest amino acid at 89.09 g/mol, is comprised of $H_2NCH_2(CH_3)COOH$ with the –COOH group being sterically available at the primary end of the molecule. The derivatization performed in this research involved a Fischer Esterification by refluxing of an amino acid with an alcohol and an acid chloride catalyst (HCl from acetyl chloride). The carbonyl of the

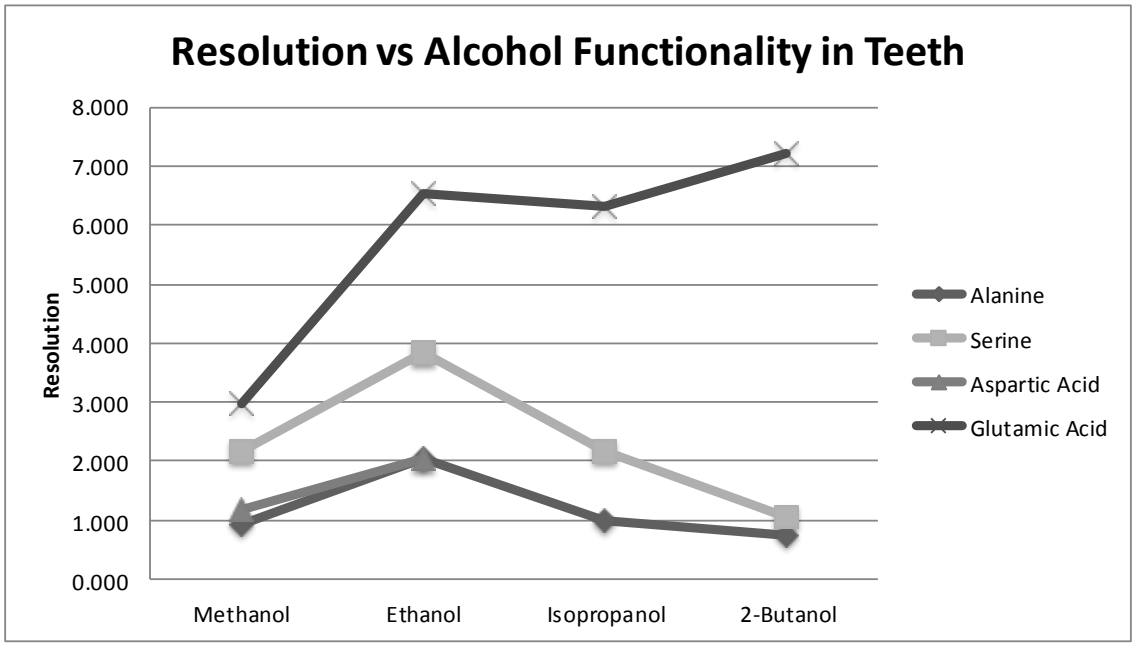


Figure 28: Graphical representation of the resolution of amino acids in tooth dentin as a function of the alcohol used during the derivatization process.

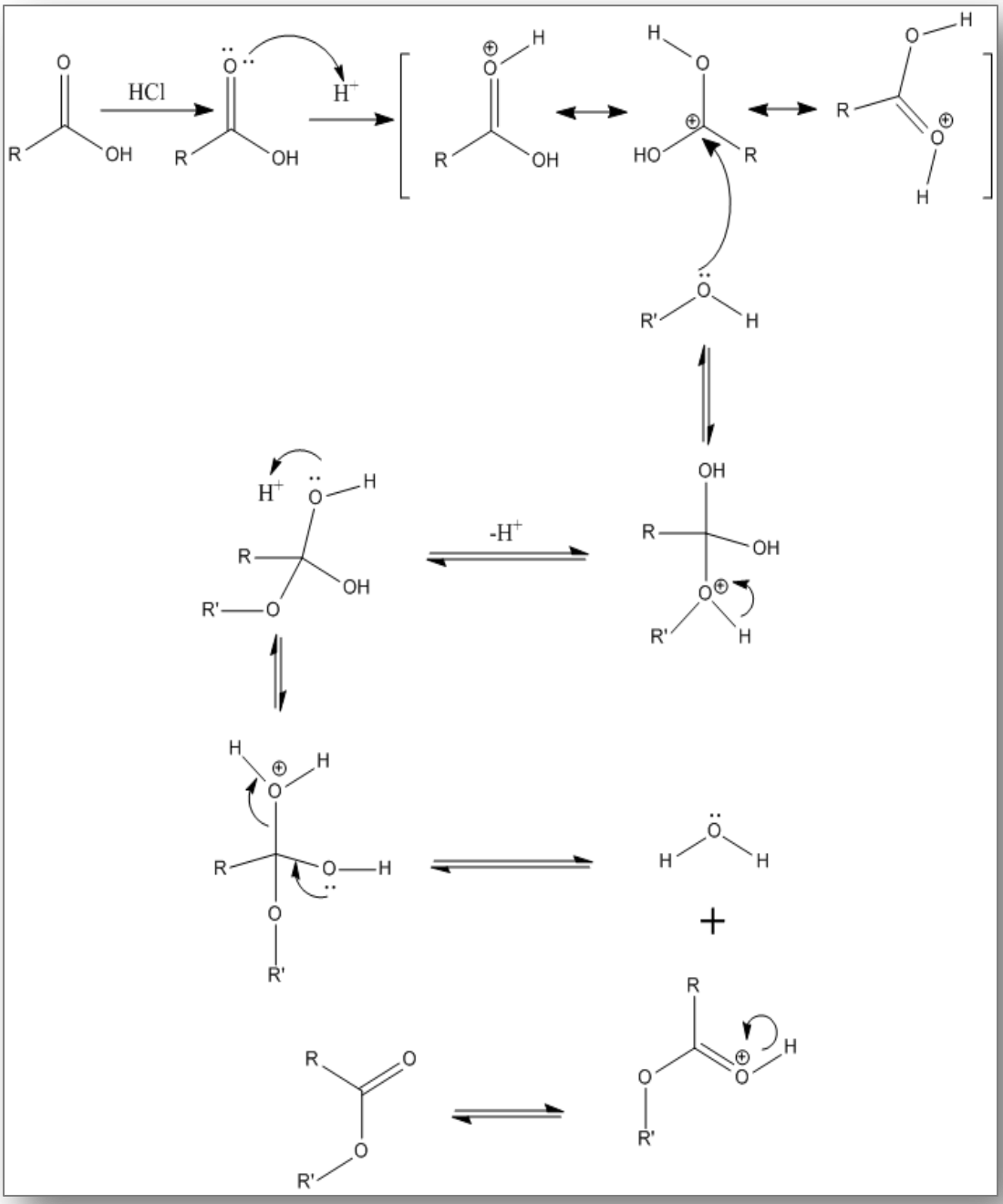


Figure 29: Mechanism illustrating the chemical reaction occurring during the esterification reaction of the amino acid with the alcoholic HCl.

carboxylic acid is initially protonated by the alcoholic HCl produced by the prior reaction of the selected alcohol and acetyl chloride (Figure 29). Through a series of resonance structures, the alcohol attacks the carbocation generated. The esterification reaction ensues as shown in the mechanism depicted in Figure 29. Since aspartic and glutamic acids are dicarboxylic acids, enough reagent was provided to ensure a complete reaction. When a bulky alcohol is used such as the isopropanol and 2-butanol, efficiency in derivatization decreases due presumably to steric hindrance.

During the Fischer Esterification, water is a by-product. To complete the derivatization procedure, acylation is required. The following step is acetylation using a highly water reactive species. The water by-product must be removed efficiently to prevent further reaction. To accomplish this, 1 mL of dichloromethane was added to the evaporated solution. Drying a second time with dichloromethane aids in the removal of any residual water.

K. Acetylation Process

Much like the esterification process, the carboxylic acid functional group is being attacked with the difference being the amine from the ester formed in the esterification process is reacting with the trifluoroacetic anhydride. Once all water is removed from the esterification step, trifluoroacetic anhydride was added to begin the acylation of the amine (Figure 30). Once this has reacted, the solution is evaporated and reconstituted with dichloromethane solvent for the gas chromatography analysis.

L. Reproducibility

In order to validate the method in this research, it was imperative to establish the reproducibility of the technique for each amino acid of study. To accomplish this, four

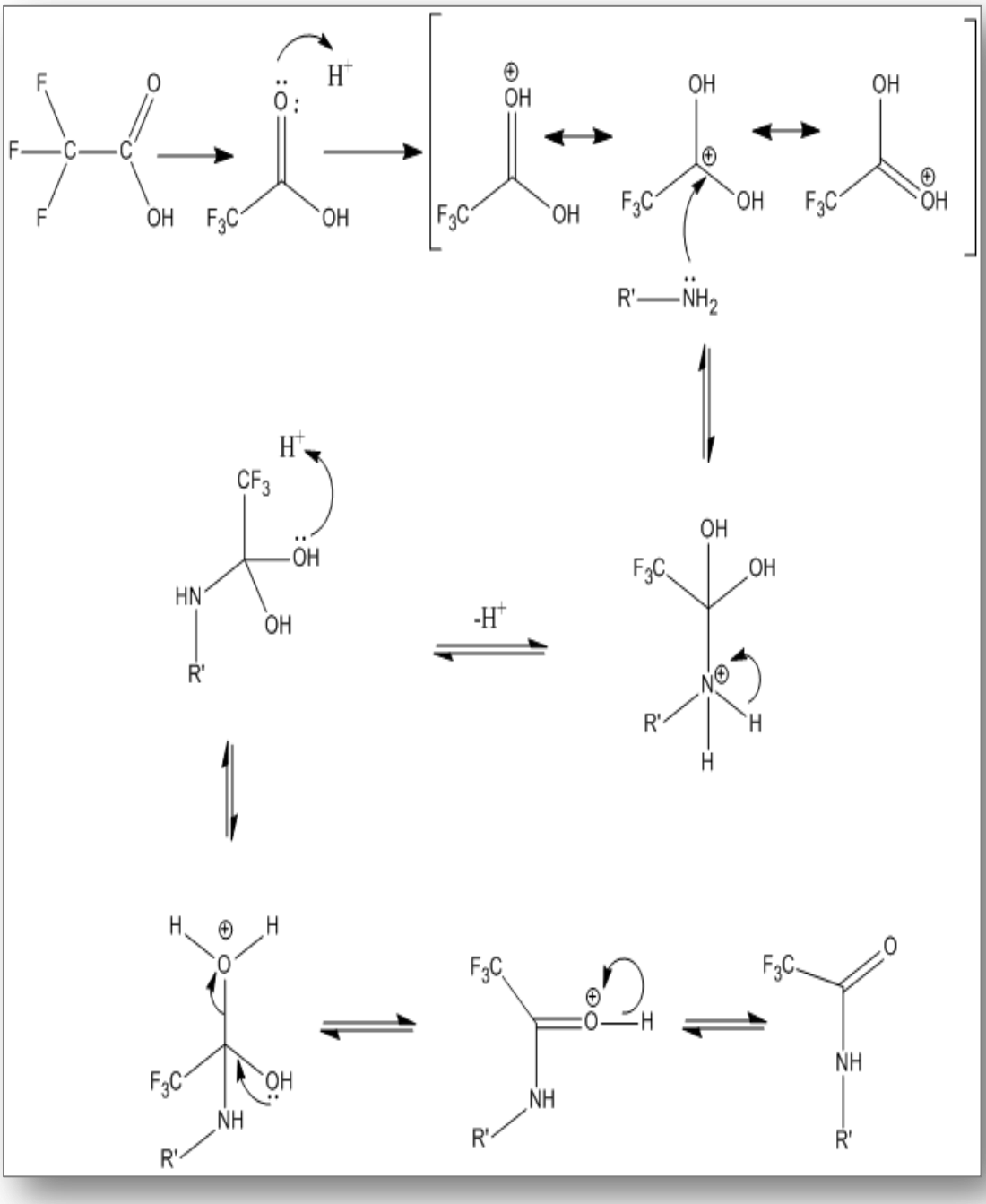

Figure 30: Mechanism illustrating the chemical reaction occurring during acylation of an amine by an acid chloride.

portions of ~10 mg from the same dentin donor was obtained. The established procedure was completed for each sample under identical conditions beginning with acid hydrolysis and terminating with the gas chromatography analysis. Once each chromatogram was produced for the four samples, peak heights for each amino acid were measured and the ratio of D/L calculated. To obtain the peak heights, a baseline for each peak was established and the peak was divided using the apex as the starting point for the division. The peak height was measured using calipers from the intersection of the divide to the summit of the peak. Each ratio was then calculated by dividing L-peak height into D-peak height. The values were entered into a spreadsheet which allowed for the grouping of the four samples by specific amino acids. The standard deviation of the D/L ratios were calculated: alanine had a relative standard deviation of 8.19%, serine 9.55% RSD, aspartic acid 18.9% RSD, and glutamic acid 15.8% RSD (Table 3). Relative standard deviation was calculated using Equation 2.

*Equation 2* $$\%RSD = \frac{Standard\ Deviation\ of\ the\ AA\ set}{Mean\ of\ the\ AA\ set}\ X\ 100$$

Each amino acid data set (AA set) was calculated individually to produce the standard deviation and the mean of the four samples.

M Ruggedness

The length of time required for the completion of the experimental process is long and arduous. Dentin samples undergoing the full experimental procedure requires approximately 12 to 13 hours to complete including hydrolysis, evaporation, isolation, and derivatization. The gas chromatography specifications were set to 55 minutes based

Table 3

**Relative standard deviations for each amino acid data set from the same dentin donor derivatized individually**

| Amino Acids | Area Ratio D/L | %RSD |
|---|---|---|
| 1. Alanine D/L | 0.0656 | 8.19% |
| 2. Alanine D/L | 0.0794 | |
| 3. Alanine D/L | 0.0692 | |
| 4. Alanine D/L | 0.0725 | |
| | | |
| 1. Serine D/L | 0.330 | 9.55% |
| 2. Serine D/L | 0.297 | |
| 3. Serine D/L | 0.363 | |
| 4. Serine D/L | 0.300 | |
| | | |
| 1. Aspartic Acid D/L | 0.176 | 18.9% |
| 2. Aspartic Acid D/L | 0.202 | |
| 3. Aspartic Acid D/L | 0.209 | |
| 4. Aspartic Acid D/L | 0.134 | |
| | | |
| 1. Glutamic Acid D/L | 0.112 | 15.8% |
| 2. Glutamic Acid D/L | 0.156 | |
| 3. Glutamic Acid D/L | 0.149 | |
| 4. Glutamic Acid D/L | 0.121 | |

on the best resolution and temperature ramping observed. Samples were often required to be run the following day due to time constraints. Therefore, the requirement exists to determine the integrity of the samples over a period of time. To accomplish this study, 5 mg of the D/L standard consisting of alanine, serine, aspartic acid, and glutamic acid underwent derivatization and subsequent gas chromatography. The D/L peak ratio was calculated using the peak heights as in the reproducibility study. The standard was maintained in an 19 °C storage devoid of any light and sealed with a Teflon cap covered in 2 pieces of parafilm to prevent solvent evaporation. Over a series of approximately one month, the standard was injected in the GC for analysis with around 3 days lapse between each injection. Each chromatogram was analyzed for the individual amino acid peak height ratios. The resultant ratios were plotted against days elapsed and the relative standard deviations calculated (Figure 31). The ruggedness test determined the integrity of the sample was maintained for up to 22 days or around three weeks. Using Equation 3, the %RSD was calculated and compared to that of the reproducibility study. It was found that alanine maintained 4.47% RSD, serine 3.23% RSD, aspartic acid 18.2% RSD, and glutamic acid 11.5% RSD. In comparison to the reproducibility data (Table 4), the relative standard deviations of the ruggedness test were slightly lower but were found to be in correlation among the two sets of data. This study illustrated utility of the sample for up to three weeks-time which eliminated the possible problem that time constraints may have caused by running the same the next day.

N. Determining Linear Correlation of Amino Acid Racemization to Chronological Age

The primary focus of this research was to not only determine an effective procedure but to apply the amino acid racemization over time as a marker for age

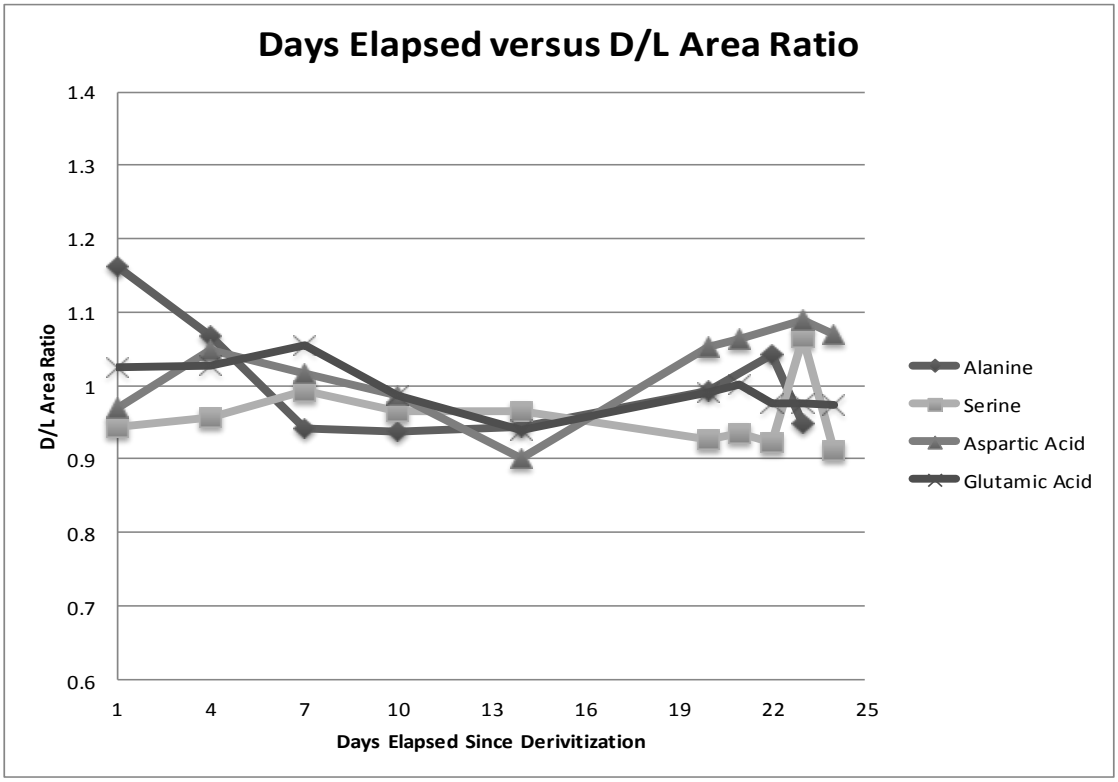


Figure 31: Ruggedness analysis illustrating the utility of the sample for up to 21 days.

Table 4

**Comparison of Relative Standard Deviation between Ruggedness Test and Reproducibility Analysis**

| Amino Acid | %RSD Ruggedness | %RSD Reproducibility | %Difference |
|---|---|---|---|
| **Alanine** | 4.47 | 8.19 | 3.72 |
| **Serine** | 3.23 | 9.55 | 6.32 |
| **Aspartic Acid** | 18.2 | 18.9 | 0.700 |
| **Glutamic Acid** | 11.5 | 15.8 | 4.30 |

determination at death. For the purpose of this study, the supposition is made the amino acids stop racemizing once they are extracted which would mirror the proposed biological cessation of racemization upon death of an individual. Each tooth sample underwent the full experimental procedure and subsequent GC analysis. The D/L ratio was determined for each sample and calculated using a linear regression analysis. Linear regression is used in part because of the linear predictor function in which a dependent variable in the y-axis can be determined based on the independent explanatory variable in the x-axis. The predictor function allows an unknown aged tooth to be placed in the data set and the explanatory variable determined. Essentially, a calibration curve is established with the linear regression calculation on the y-axis and the known age on the y-axis. Equation 3 illustrates the linear regression equation used to determine the y-axis data.

*Equation 3*

$$[Ln(\frac{(1 + {}^{D}/_{L})}{(1 - {}^{D}/_{L})})]$$

where D/L = Peak Height D-Amino Acid ÷ Peak Height L-Amino Acid

The most established amino acid as an age marker is aspartic acid based on previous research. To ensure this research was able to reproduce this convention, aspartic acid was evaluated first. The age range sampled for aspartic acid began at 12 years of age and progressed to 66 years of age. The ratio of D- to L- aspartic acid was determined and the resultant ratio calculated for linear regression using Equation 4. Subsequently, a plot was made (Figure 32) and the correlation established. Aspartic acid was found to have a tendency toward linearity with a correlation coefficient of $9.112 \times 10^{-01}$.

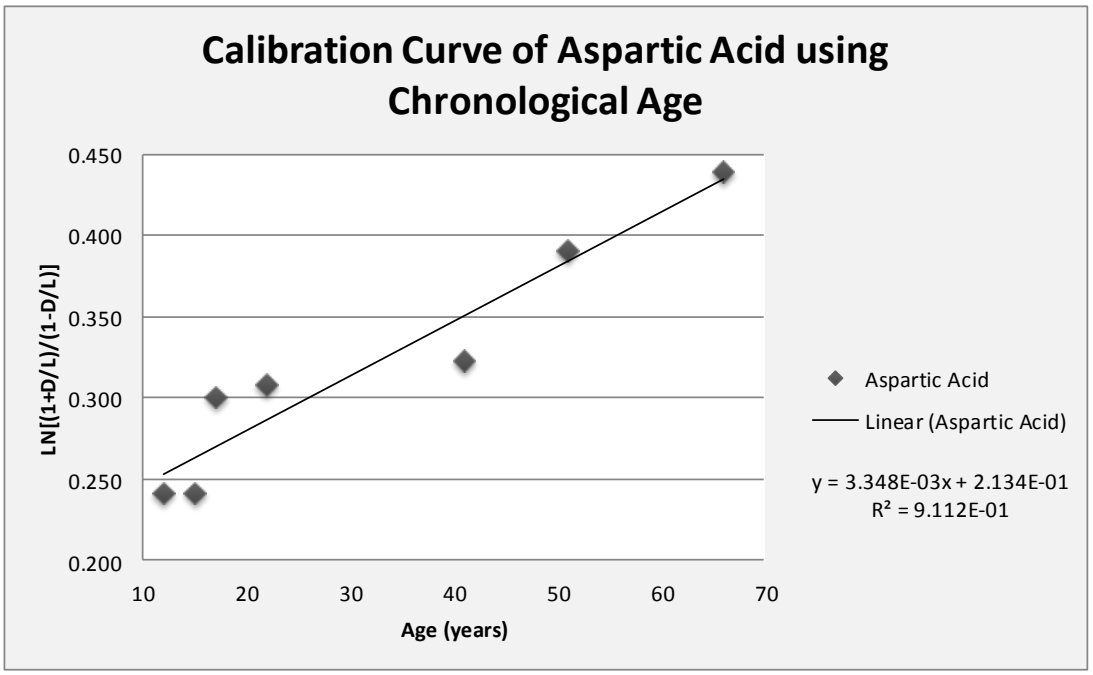


Figure 32: Aspartic acid calibration curve for age determination using linear regression analysis.

The age correlation graph was generated using 8 dentin samples from 3 teenagers, one 22 years old, 41 years old, 51 years old, and 66 years old. While the age curve establishes a wide range of ages, it is based upon the number of donated samples available to the research.

Another goal of this research was to evaluate other amino acids as possible age identification markers. Such is why alanine, serine, and glutamic acid were included in this study. Alanine and serine proved to be inadequate as age identification markers. This was determined for a host of reasons including resolution and recovery, variance in ratio calculations, or the inability to complete a calculation. Alanine was found to have adequate resolution at the specifications for the GC in the standards as well as dentin sample. However, over the range of dentin samples, alanine was found to be unidentifiable for the D- conformation. There appeared to be no consistent changes in the chromatograms over the ages but rather the complications emerged randomly. Due to the random nature of this amino acid's complications, it was impractical to attempt usage of alanine as an age identification marker. Serine too had complications in which the D- and L- peaks were often indistinguishable. This too occurred randomly with alanine in that well recovered peaks would be available but would also be missing in chromatograms in teeth of the same age range. The well recovered peaks offered promise due to large baseline resolution but were too inconsistent to apply as an age identification marker.

Glutamic Acid was the final amino acid determined for this study. Like aspartic acid, D- and L- peak heights were measured and the ratio calculated. Using Equation 4, the linear regression was calculated and a plot was made against chronological age

(Figure 33). Glutamic acid had six samples beginning at age 12 and ending at age 73. The correlation coefficient was established for this amino acid and found at $9.792x10^{-01}$. Like aspartic acid, glutamic acid displayed a tendency toward linearity. To further establish this amino acid as a credible age identification marker, more samples would be required for statistical significance.

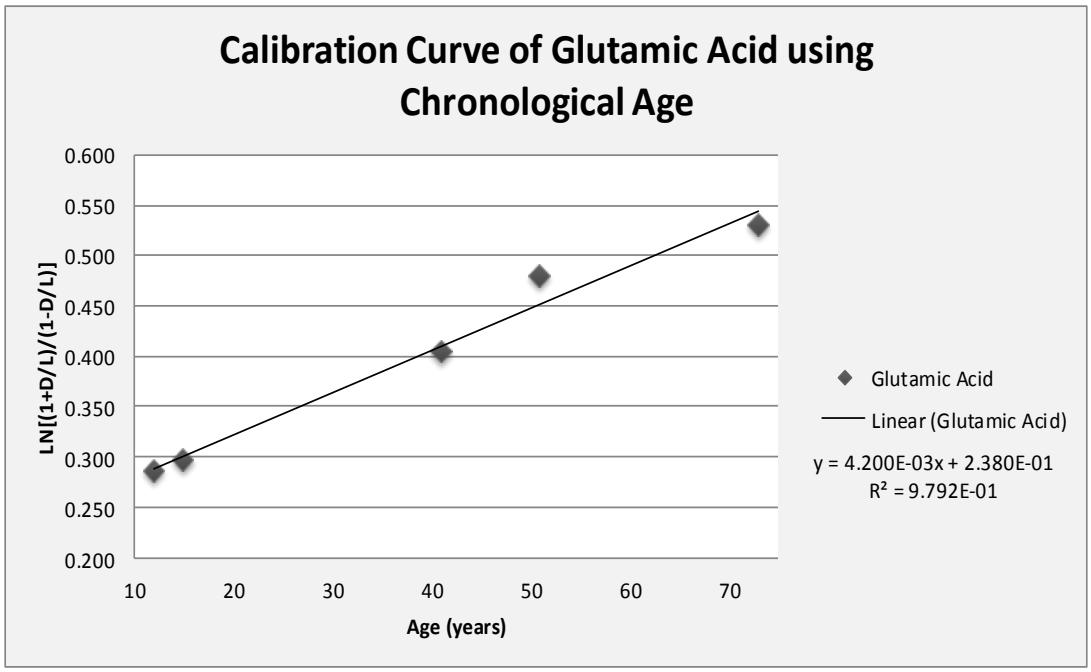


Figure 33: Glutamic acid calibration curve for age determination using linear regression analysis.

## IV. Conclusions and Future Research

Based upon the study conducted for the most effective esterification alcohol, ethanol provided the highest recovery as well as baseline resolution of the amino acid peaks. This was demonstrated for both amino acid standards as well as amino acids derived from dentin. Dentin was found through SEM-EDX to contain a higher intensity of carbon content than the more inorganic content found in the enamel. This established the media in which the highest proteinaceous material housing the amino acids of interest was located and thus targeted for extraction. A successful procedure was implemented to remove organic contaminants on the exterior of the tooth prior to cutting. Likewise, an efficient means of dentin isolation and pulverization was established using a Dremel drill outfitted with a diamond blade to remove the top crown layer and expose the interior dentin layer. Dentin extraction was most successful using a Dremel drill with a diamond tip drill bit that excised primarily dentin and created an immediate powder requiring no further pulverization. Isolation of the amino acids was completed on a cation exchange column with a determined volume between 2.0 and 3.0 cm for effective recovery and clean-up from the matrix.

Reproducibility of the experimental method was established with the relative standard deviations of all amino acids being below 20% RSD. The integrity of the sample was evaluated and found to remain viable for up to three weeks. The relative standard deviation of the ruggedness test which determined the integrity was found to correlate with the reproducibility study for the amino acids. Likewise, the relative standard deviation for the ruggedness test was determined for all amino acids to fall below 20% RSD. Aspartic and glutamic acid were established to present a tendency

toward linearity with high correlation coefficients. However, more samples would need to be attained to further establish the linearity or lack thereof.

Future research is vast and there are numerous avenues to explore. Environmental effects such as pH, humidity, and climatic differences are of great importance. For this study, cessation of racemization was assumed due to the removal of the tooth under medical conditions. However, when considering the utility of the research, it is prudent to establish these conditional effects on racemization. Human remains can be discovered in a variety of climates, conditions, situations, and possibly many years after the completion of decomposition. Each factor may affect the biological racemization. Temperature has been established as a factor to increase the rate of racemization as well as various pH environments. Future research will establish these conditional effects on racemization and subsequently the variance it creates in age determination using a linear predictor model. Future plans for the research are to evaluate Differential Scanning Calorimetry and Thermal Gravimetric Analysis and compare this to mass spectrometry readings at specific temperatures. This will establish the loss that is occurring from the dentin sample based upon the temperature in which the loss occurs.

A further goal of this research is to transition from gas chromatography to capillary electrophoresis. As previously mentioned, the length of time required to complete the derivatization process is long and arduous. The amino acids are soluble in water and capillary electrophoresis would prove to be a more “Green Chemistry” method using aqueous solutions instead of toxic organic solvents. Capillary Electrophoresis also

provides comparable resolution to capillary gas chromatography as well as typically faster elution times.

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