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Dipeptide Synthesis and the Age Determination of Bones

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Abstract: **Age determination of the skeleton, based upon the aspartic acid racemization technique and the radiocarbon dating technique were carried out. Previous areas of analysis were based upon the morphology of the skeleton but this proved to be imprecise at the best of times. The chemical analysis has shown more precise and reproducible results. In this study, I looked at two complementary techniques that could be used to estimate the date of birth, the date of death and the age of an individual, who has been found at the scene of a crime, etc. In a case study forty-four teeth from a group of participating individuals were analysed using the aspartic acid racemization technique, to analysis the tooth crown dentin and the radiocarbon analysis of the enamel. The radiocarbon analysis showed good precision and had an overall absolute error of 1.0 ± 0.6 years. The aspartic acid racemization technique also had good precision and showed an absolute error of 5.4 ± 4.2 years. The radiocarbon analysis gives the investigator estimation as to the year the individual was born and the aspartic acid racemization analysis gives the investigator a good estimation as to the age of the individual at the time of their death. With the help of these two techniques, it is possible to determine a date of death for the individual, especially if they are an unidentified individual. The conclusion of the work explains how these techniques are a great asset to the Forensic community and how they are now a crucial part to most of the investigations conducted on a daily basis, especially those of massive proportions.**

Keywords: **amino acid racemization, dipeptide synthesis and age determination.**

I. INTRODUCTION

All dipeptides and proteins are constructed from a chain of amino acids, in a variety of lengths. Dipeptides have the shortest chains possible, consisting of just two amino acids. Proteins are made of longer chains and consist of more than two amino acids. All these amino acids are joined by the formation of an amide or peptide bond. Within the natural environment, there are many dipeptides with an array of functions, although it is possible for them to be generated within a laboratory environment. (Shawn, D. 2002, Hart, 2007) Dipeptides can be used in many different areas, such as commercially and industrially, as well as, playing a vital role within the biology of many of Earths species. Amino acids are the main area for study but many researchers will also look into the area of dipeptides so that they may gain a better understanding and knowledge of them and their function. More research can be done by looking into dipeptides, which are already known so that they can be better understood as well. (WISEGEEK, 2011)
In order for the amide bond or peptide bond to be formed a condensation reaction (the loss of a water molecule) between the amino group of one amino acid and the carboxylic acid group of another amino acid must occur.
The reaction scheme of the formation of the peptide bond is as follows:

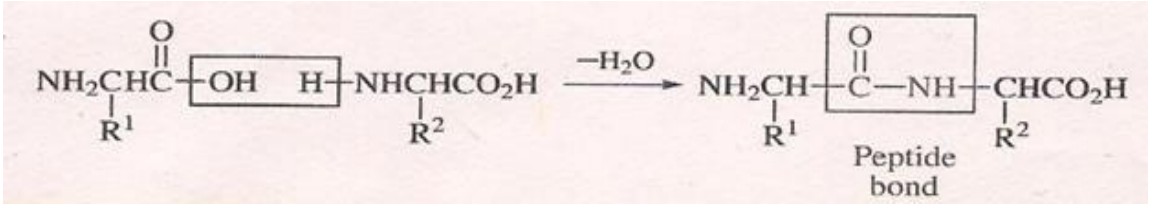

Figure 1: Peptide bond formation; (Shawn D. 2002)

Once the dipeptide has been constructed, an N-terminus and a C-terminus are clearly visible upon the dipeptide. The diagram below illustrates both terminuses.

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Figure 2: N and C-terminus present upon a dipeptide (Life Protein. 2014).

The term "protein" was derived from the Greek word *proteios*, meaning "primary". This word came amino acid units in a variety of sequences. Proteins are composed of α-amino acids (which are amino acids that have been obtained from the hydrolysis of proteins) and carboxylic acids, which have an amino group on the α-carbon atom. (Hart, 2007) Proteins consist of peptides, which are long molecular chains. Synthetic peptides are used, mainly, as drugs (as they are biologically active) or in the diagnosis of many diseases. It is difficult for peptides to be synthesised as the synthetic chemist has to ensure that the amino acids which make up the chain are added in the correct order and that they do not undergo any other reaction. These peptides are folded in a particular way, or are made from several peptides folded together. (Atherton el at., 1989) An example of a α-amino acid is shown in figure 3 below. into existence before anything was known about the chemical structures of any of the natural macromolecules. (Elmore, 1968) Proteins are the key molecules in the process of life as they carry the instructions for making different organisms, they are polymers, which occur naturally.

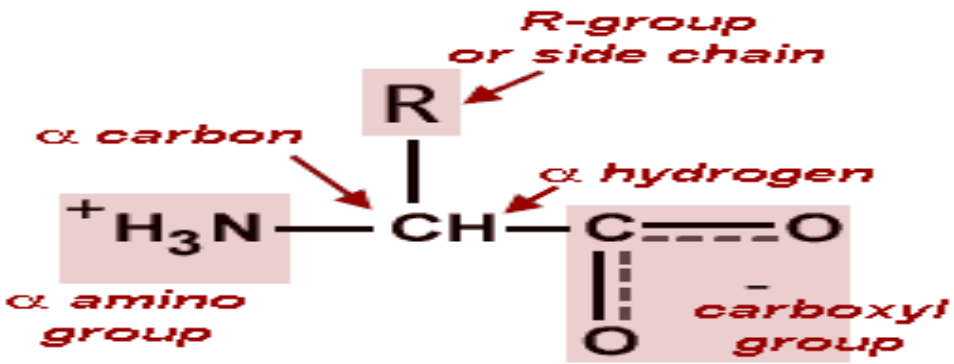

Figure 3: A α-amino acid; (Biochemistry Department, University of Arizonia, 2003)

Peptides have a wide range of interesting and important biological activities. (Shawn, D. 2002) They are chains (which have a variety of lengths) of amino acids which are linked by amide bonds and are the principle make-up of proteins. The linking of these amino acid chains is an acylation reaction (which is the addition of an acyl group). The synthesis of peptides occurs very rapidly within living cells, but could only be synthesised artificially by a process, which was long and very slow. A new technique known as Solid Phase Peptide Synthesis (SPPS) has been used to construct these synthetic peptides. There are two main uses of synthetic peptides: they are as peptide drugs and as peptides for diagnostic purposes. (Atherton el at., 1989) Within each protein, there are a number of amino acids. Upon some of these amino acids, there are side chains, which help to differentiate the amino acids from one another. These peptide chains are synthesised by building them up one unit at a time with the amino acid units. (Elmore, 1968) There are twenty amino acids, which are commonly used to construct all peptide chains and form the much larger proteins.

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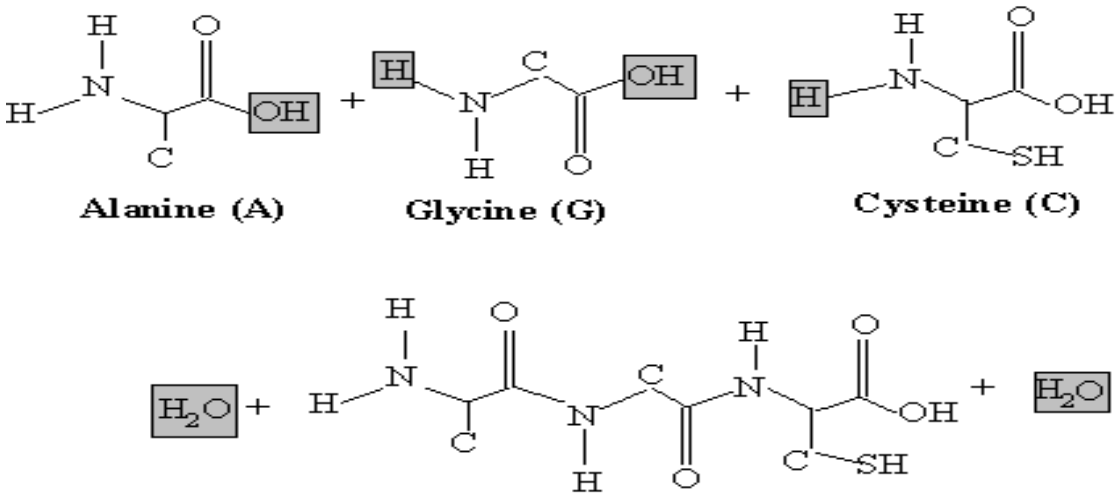

Figure 4: A peptide chain consisting of Alanine, Glycine and Cysteine and two water molecules (Karchin, 2009)

Blocking agents are used to prevent amino acids from reacting with themselves, before the formation of the amide or peptide bond of the dipeptide can be achieved. Another reason why a blocking agent is used is to reduce the chance of racemization occurring during the synthesis. These amino groups can be converted easily into free amines and they can be useful in Merrifield solid-phase peptide synthesis. (Hart, 2007) The blocking agent joins to either the carboxylic acid of the amino acid or the amino group of the amino acid so that the opposite active group of the amino acid is free to react with the new amino acid. (Zinelaabine et al. 2012) The most commonly used N-protecting groups are t-butoxycarbonyl (Boc) groups, especially in solid-phase peptide synthesis. These amine and amino acid reactions are conducted under aqueous or anhydrous conditions by a reaction with a base and the anhydride Boc_2O. (Hart, 2007) Once the polymer has been attached to the amino acid, the protecting group is removed. The removal of the protecting group is achieved by a reaction with acid, under mild conditions. (Hart, 2007)

A dipeptides synthesis from its constituent amino acids involves the formation of a peptide bond (–CO.NH–) so that the correct sequence of amino acids is made and so that racemization does not occur at the asymmetric α-carbon atom. This point does not occur with the amino acid glycine. This process is more complicated than it sounds, as you are not simply forming an amide bond by mixing the desired amino acids together in a test tube. (Atherton el at., 1989) Within solutions which contain a mixture of two amino acids, four dipeptides could potentially be formed. To ensure that the desired dipeptide is the only one formed, the basic group of one amino acid and the acidic group of the other must be unable to react with one another. This "deactivation" of the amino acid acidic and basic sides if known as the protection of the reactive groups. These groups will further more be known as protected groups. (Bodanszky, 1993) As mentioned before in section 1.2 above, protecting groups have to be added to certain sides of the amino acids to prevent them from reacting with themselves and creating a mess. The steps below indicate the synthesis of a dipeptide:

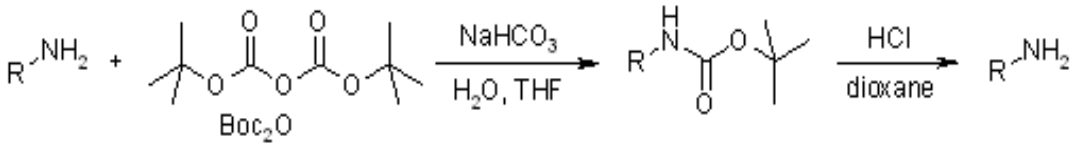

Figure 5: Protection of a polymer with di-*t*-butyl dicarbonate; (Hart, 2007)

1. Once the amino acid has been attached to the polymer, the protecting group is removed by a reaction with an acid under mild conditions. The next amino acid also has to be protected. The deprotection by-products are gaseous (2-methylpropene and carbon dioxide), which are easily removed from the reaction mixture.
2. Once the amino acid has been attached to the polymer, the protecting group is removed by a reaction with an acid under mild conditions. The next amino acid also has to be protected. The deprotection by-products are gaseous (2-methylpropene and carbon dioxide), which are easily removed from the reaction mixture.

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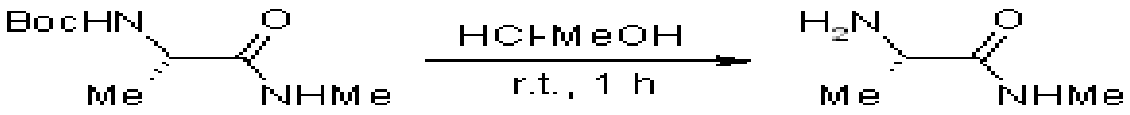

Figure 6: Deprotection of an amino acid; (Hart, 2007)

3. A new N-protected amino acid is linked to the first amino acid. The linkage of these two amino acids is achieved with the aid of dicyclohexylcarbodiimide (DCC) as a catalyst. The dicyclohexylcarbodiimide is reduced during this reaction to 1,3-dicyclohexylurea (DCU).
4. Once the desired amino acids have been joined in the proper sequence and the N-terminal amino group has been deprotected, the completed dipeptide is detached from the polymer. The side-chain protecting groups are also removed at this stage of the reaction (if side-chain protecting groups are present).

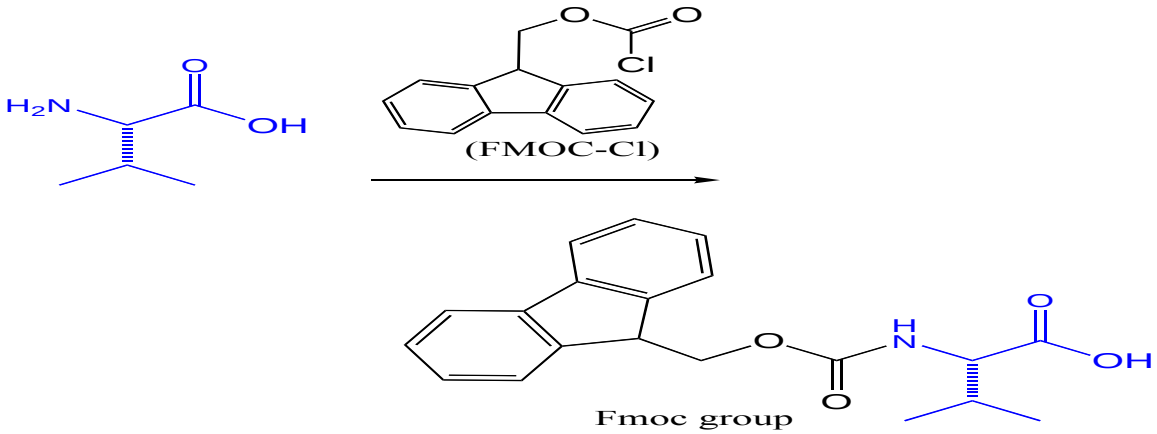

Figure 7: Solid-phase synthesis of a Dipeptide; (Hart, 2007)

There are three main problem associated with the synthesis of dipeptides and even peptides. These three problems are:

1. That the reactions will be non-quantitive
2. That there will be incomplete orthogonality between the temporary and permanent blocking or protecting groups;
3. And finally, that the side-reactions will occur especially at the complete deprotection stage of the reaction and at the cleavage from the carrier stage of the reaction.

If any of the *N*-terminal residue of the peptide-polymer conjugate remains unacylated after any of the coupling stages, then this will lead to contaminated of the end product with sequences which are incomplete.

II. PROTECTING GROUPS

There has been a great amount of effort spent over the last 70 years on developing protecting groups for peptide synthesis. Amino acids can undergo other reactions which are typical of carboxylic acids or amines. E.g a carboxyl group can undergo an esterification reaction.

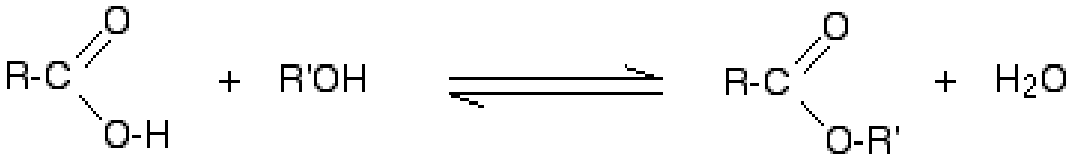

Figure 8: Esterification of a carboxyl group; (Hart, 2007)

The amino group can undergo an acylation reaction to an amide.

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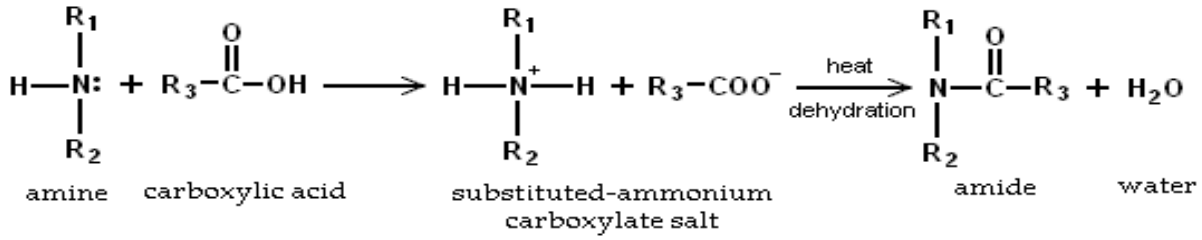

Figure 9: Acylation of an amino group to an amine; (Hart, 2007)

These reactions are useful in temporily protecting either of the functional groups, during the linking of amino acids to form peptides or proteins.

Amino Group Protection- One of the most important advances in peptide synthesis was made in 1932 by Bergmann and Zervas, when they intorduced the benzyloxycarbonyl protecting group. The benzyloxycarbonyl amino acid essential point is that, as an ester of carbamic acid, the nitrogen atom does not have any nuclephilic properties and so will not take part in the peptide bond formation. Another important aspect, is that the protecting group is removable under a variety of conditions where the peptide bond is not affected. The development of an improved carbamate protecting group was also made during this time, in the form of *t*-butyloxycarbonyl (*t*-Boc). T-butyloxycarbonyl is illustrated in figure 10 below. This group is easily removed by treating it with aqueous trifluoroacetic acid.

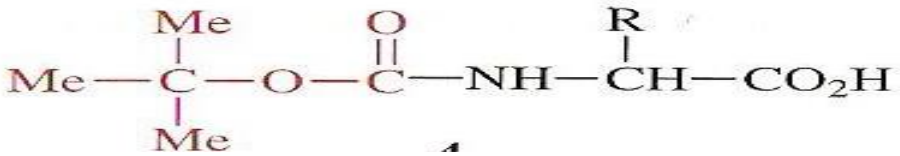

Figure 10: A *t*-butyloxycarbonyl molecule;

The avability of the two protecting groups mentioned above, removed under different conditions provides a stragedy for peptide synthesis which contains the amino acid lysine. A further improved in this area of protecting groups was made by Carpino, who introduced the 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group. 9-fluorenylmethyloxycarbonyl is illustrated in the diagram below.

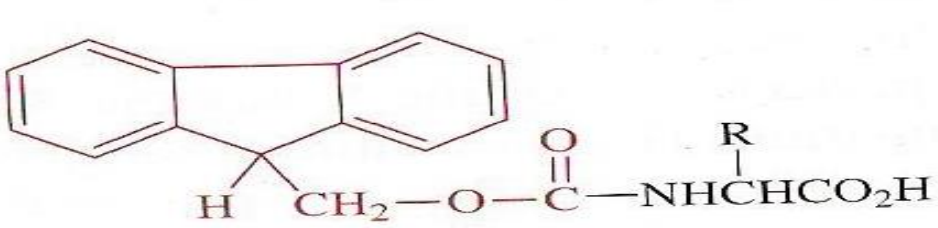

Figure 11: A 9-fluorenylmethyloxycarbonyl molecule; (Hart, 2007)

An advantage of this protecting group is that it is removable under very mild conditions by treating it with an organic base.

Carboxyl group protection- Carboxyl grooup protection is usually carried out by concersions to esters. During the process of peptide bond formation, these ester must be unreactive and easily removed during the stage of elongation or during the stage of the formation of the final deprotected product. Two of the most widely used derivatives are benzyl esters and *t*-butyl esters. Both these derviatives are illustrated below.

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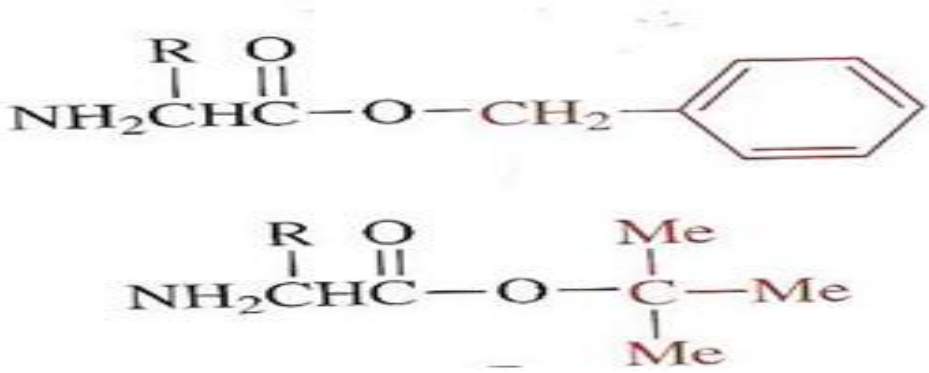

Figure 12 and 13: Top image; Benzyl ester molecule, bottom image; *t*-butyl ester molecule; (Hart, 2007)

As mentioned above for the amide group protection section, it is important to have two protecting groups which can be removed under completely different conditions, as there is a need for differential protection of the amino acids with carboxylic acid side chains.

Side-chain Protection- The side chains which are always protected are cysteine, serine, threonine, tyrosine, histidine and arginine. When choosing a side chain protecting agent, it will depend upon the protecting group used for the α-amino function, since it is important that the side chains remain protected when the N-terminal amino acid is deprotected for chain elongation.

III. AMINO ACIDS IN FORENSIC SCIENCE AND AGE DETERMINATION

Identifying victims of a crime or of a mass disaster has become easier through the analysis of amino acids, such as aspartic acid. The determination of age of these victims is an important setting of a crime scene or of a mass disaster as the death, date of birth and year of death, as well as the gender can be a guide to the investigators, as to the correct identity of a person, especially if there are a large number of possibilities. An accurate determination of age can be achieved from the combination of two techniques. These techniques are the analysis of aspartic acid and carbon-14 dating. At a crime scene or a mass disaster, the investigator's will analysis DNA so that they can determine the gender of the victim or victims but they cannot determine the age of the victim or the year that the victim died. The popular forensic technique of analysing amino acids such as aspartic acid gives a good estimation of age as it transform very quickly. When an analysis of tooth enamel with aspartic is carried, the results that will be obtained will be an approximate age at the time of death. (Greenwood, 2013)

IV. AMINO ACID PRESENT IN PROTEIN

All the amino acids which are present within a protein, except the simplest amino acid glycine, have a minimum of one asymmetric carbon atom which can exist as one of two stereoisomers. This means that the chemical groups are arranged in two different ways in space. When a single asymmetric carbon is present, the two different forms are known as optical isomer. There is very little difference between these stereoisomer's chemically, but biologically, they are very different. The common means for these two forms are L – amino acid and D – amino acids. The plane – polarized light rotation direction of these amino acids depends upon the L and D designation of the amino acid. The stereoisomers are mirror images of one another so cannot be superimposed upon one another (Duane. 2000).

The majority of the amino acids within protein are L – amino acids. They slowly change to the D – form so that equilibrium is reached. This is illustrated thus:

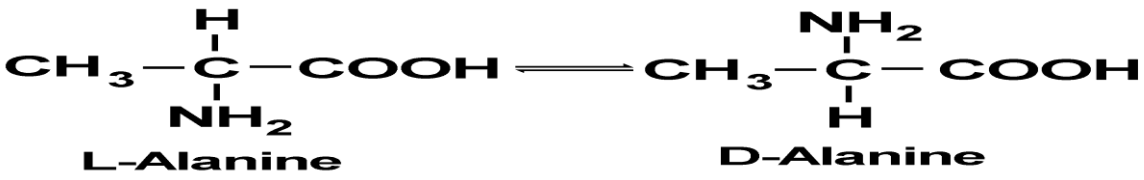

Figure 14: A mixture containing equal amount of L – and D – forms. (Duane. 2000)

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These amino acids make up the back bone of peptides, in repeating sequences of one nitrogen and two carbon atoms.

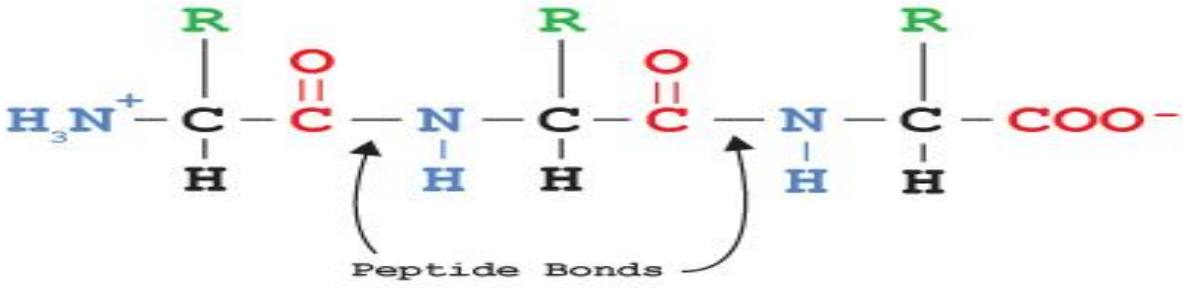

Figure 15: peptide chain containing amino acids; (Hart, 2007)

As the chains of amino acids get longer they begin to fold and twist in on themselves, eventually creating 3D molecules. It is the effect of this folding and twisting that proteins have levels of structures. The chain of amino acids, which is illustrated above, is known as a primary structure of proteins. This structure is the first level of the protein structures. The second level of protein structures or secondary structure, is a polypeptide chain which coils about itself in a spiral manner to form a helix, which is held rigid by intrachain hydrogen bonds. This structure is known as the α-helix.

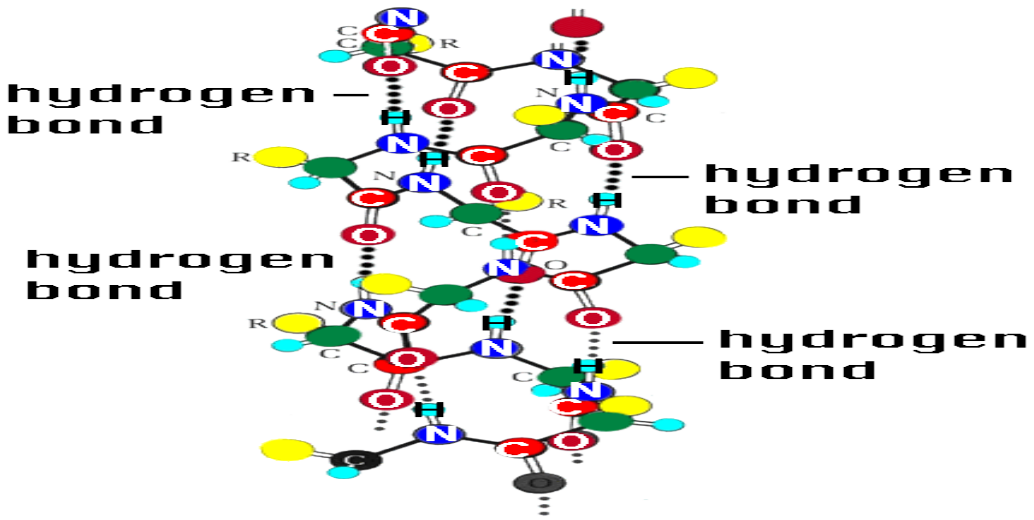

Figure 16: A α-helix structure; (Nishiura,J.1999).

The other arrangement for secondary is the pleated sheet arrangement of the protein. Within the pleated sheet, the peptide chains lie side by side and are held together by hydrogen bonds which are interchain.

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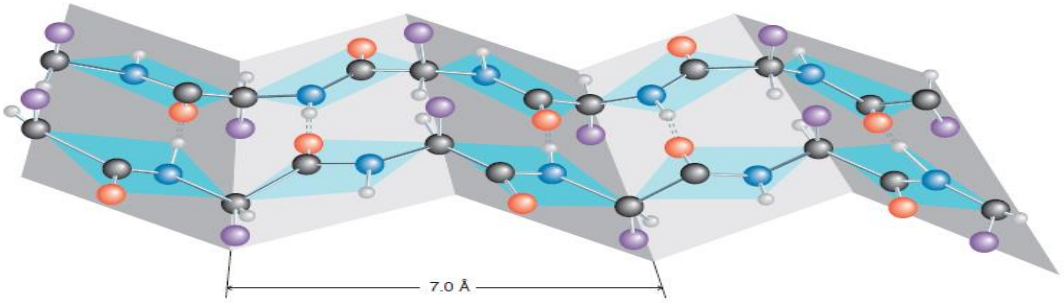

Figure 17: A pleated beta sheet; (Gaur, 2014).

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The tertiary structure of a protein is a 3-dimensional shape where the whole chain folds in on itself, including the secondary structure.

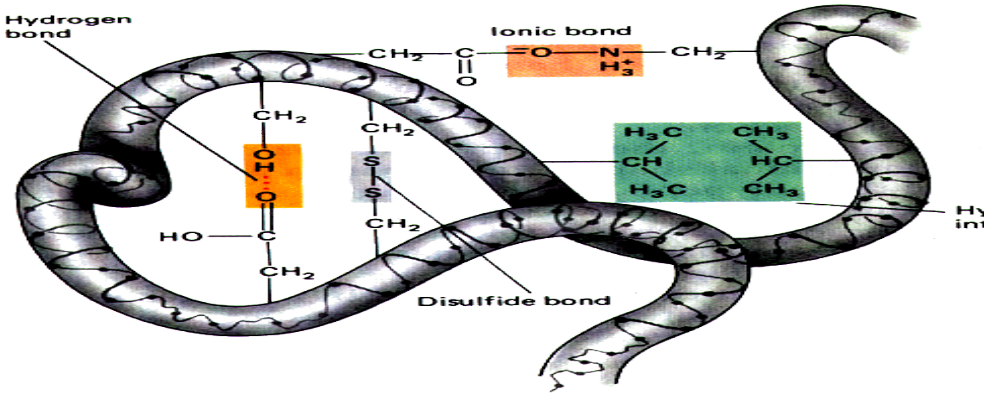

Figure 18: Tertiary Structure of a Protein; (Nishiura,J.1999).

The structure of a quaternary protein contains the clustering of several individual peptide chains into a final specific shape. There are a variety of bonding interactions within the structure, which include hydrogen bonds, salt bridges and disulphide bonds. All these bonding interactions hold the various chains in a particular geometry. Proteins with quaternary structures are split into two major categories, which are fibrous and globular. (Ophart, 2003).

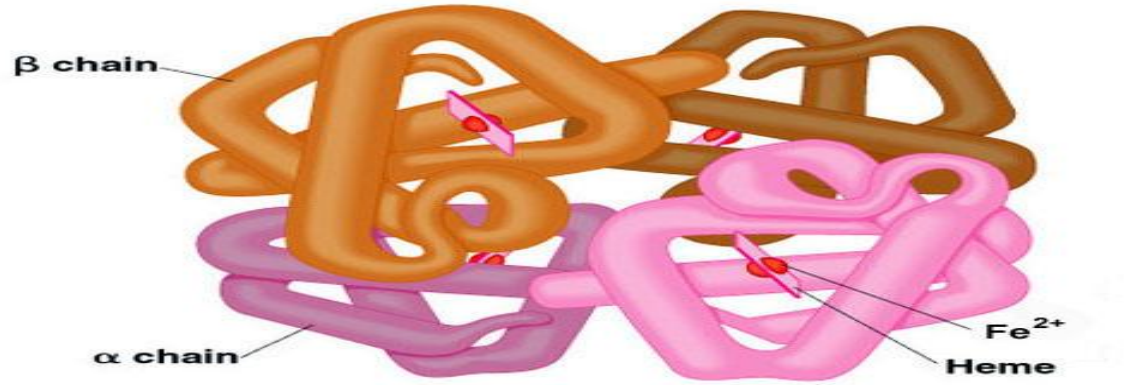

Figure 19: Quaternary structure of a peptide; (King, 2013).

V. RACEMIZATION PROCESS IN DATING

By definition racemization is "*a chemical reaction in which an optically active compound is converted into a racemic mixture*." Racemization is the name given to the process which changes an L – amino acid into a mixture of the L- and D – forms. These amino acids are found in fossil shells, bone and teeth. It was found that the older the fossil the higher the D/L ratio was going to be and that after a certain age; the amino acids were going to occur in their racemic form. The completion of the racemization process occurs when equal amounts of the L – and D – form of the amino acid are produced. There is one factor which complicates this process and this factor is that some amino acids have two asymmetric carbon atoms and so the amino acid could potentially exist in four different forms. This is known as diastereoisomers. The two mostly commonly found amino acids within peptides are isoleucine and threonine. The racemization of L- isoleucine is almost exclusively to as single form called D–alloisoleucine. Normally the separation of an L – Amino acid from its D – form is quite difficult but the separation of the L – isoleucine from the D–alloisoleucine is fairly easy. Due to this easy separation, there is a special interest in the amino acid racemization dating system of the racemization of the L- isoleucine to the D–alloisoleucine. (Duane. 2000). The extent of the racemization process could serve as a dating method, as the amino acids within a dead plant or animal spontaneously change to a mixture of its L-

form and D-form. This means the older a fossil shell or bone is, the greater the extent of racemization of the amino acids within the proteins of the shells or bones. (Duane. 2000). Some of the factors which can bring about errors in the analysis of age determination in bones are; temperature, pH, soil composition and various contaminants.

In an experiment carried out by Hare and Mitterer 1969, they measured the rate of racemization of L-isoleucine to D-alloisoleucine within a shell fragment which was heated in water at a high temperature and extrapolated the data to lower temperatures so that an estimation of the rate of racemization of L-isoleucine within the fossil shells could be determined as an approximate age for the fossil shells. (Duane. 2000).

The amino acid racemization method for the dating of marine sediments was reported later by Bada and his co-workers (Bada et al 1970 and 1972). Bada and his co-workers applied this method to the dating of fossil bones (Bada 1974, and Bada et al 1974) as well as applying amino acid racemization rates to the determination of past temperatures by measuring the extent of the racemization within several radio carbon dated bones (Schroeder and Bada 1973). The rates of racemization of amino acids in marine sediments have been measured by Kvenvolden, Peterson and brown (Kvenvolden et al 1970). Further reports of the rate of racemization of amino acids to the dating of marine sediments have been made by Wehmiller and Hare. (Duane. 2000).

The rate of racemization in marine sediments was a study conducted by Bada and Schroeder 1972, which under laboratory conditions was determined by the heating of the sediments with sea water in sealed ampoules at a range of temperatures from 100°C to around 150°C over a variety of time lengths. The extent of the conversion of L-isoleucine to D-alloisoleucine was determined by the hydrolyzation of the material in 6 molar hydrochloric acid. The rates that were obtained from those temperatures were extrapolated to 2°C and 4°C, the present average temperatures were the deep-sea cores containing the sediment samples were obtained. (Duane. 2000).

The data is believed to yield the rates at which the L-isoleucine is converted to D-alloisoleucine in the sediment through geological time. The extent of conversion of L-isoleucine to D-alloisoleucine in the core sediment samples from varies depths were then determined and conclusions based on the above rates were used to estimate the ages of the sediments from varies core depths. (Duane. 2000).

The bone studies were carried out in a similar way (Bada 1972). In the Arizona desert bone fragments were recovered which were believed to be between 2-3 years old. These fragments were sealed within glass ampoules and heated at a variety of temperatures. The extent of racemization of the L-isoleucine to the D-alloisoleucine was determined from the hydrolyzation of the bone fragments with 6 molar hydrochloric acid. The lower temperature rates were estimated on the bases of the rates at the elevated temperatures. Ages were calculated from a combination of conclusions that were based upon these rates. Some of these conclusions were the actual extent average temperatures at which these fossil bones are believed to have existed. The rate of racemization of aspartic acid was used in other works (Bada and Protsch 1973, and Bada et al 1974). (Duane. 2000).

Temperature is the condition upon which the rate of racemization is dependent upon. With the study of bone an uncertainty of ±2° would yield an age that has an error margin of ±50%. The introduction of further uncertainties are made possible by the risk of contamination of the fossil with any amino acids which have come from the environment freely and the change that racemization may have occurred during the acid hydrolysis process of the proteins within the fossil. The apparent age of the fossil would be the proteins within the fossil. The apparent age of the fossil would be reduced by the introduction of the amino acids which have come from recent material and would undergo little racemization. This is the contamination that the first uncertainty is speaking of. For the apparent age to increase then racemization would have to occur during the acid hydrolysis process. (Duane. 2000).

Effects which are caused by the nature of the neighbouring amino acids could cause racemization of individual amino acids during the acid hydrolysis process (Manning and S. Moore 1968). The necessary preparation step of hydrolysis of the protein can cause the apparent age to be older than the actual age. (Duane. 2000).

Serious errors within the results would not normally be caused by amino acid racemization dating method effects above, except by the error which is introduced by the uncertainty of the temperature. (Duane. 2000).

A difficult problem to an investigator is the identification of human bodies especially when there are no clues as to their identity. A crucial step for investigators is the determination of the age and gender of the human body, as these characteristics can be used to limit the search within lists such as missing persons and therefore minimize any efforts of alternatives. Although gender can easily be determined through DNA analysis, the determination of age is not as straightforward. The age determination of children and adolescents is slightly easier then the age determination of adults

(in adults it is less accurate). The younger generations age can be determined based upon methods of morphological, such as, radiological examination of the development of the skeleton and teeth. The current methods of the estimation of age include simple, less precise methods as mythological (evaluation of dental or skeletal morphology) or the complex, more accurate methods within the laboratory (radiocarbon dating of tooth enamel or aspartic acid racemization in dentin or tooth enamel). Both of the methods mentioned above result in a high precision for the estimation of age. From an analysis based upon morphological methods the error margins of age determination are greater than ±10 years but with the aspartic acid racemization method (which is more precise) and the radiocarbon analysis report that these methods have accuracies of ± 3 and ±1-2 years, respectively. Based upon this knowledge it is clear why the aspartic acid racemization and radiocarbon dating methods are currently the methods of choice for the determination of age. The method of aspartic acid racemization was discovered in 1975 by Helfman and Bada, and from this time of discovery, this method has been one of the most widely used in Forensic Science for the estimation of age. The racemization of aspartic acid involves the conversion of the L-form to the D-forms. At a temperature of 25°C, the amino acids which are present within living tissues would undergo a complete racemization of the L-form to the D-forms in approximately 100,000 years. From this time frame for the racemization it is easy to estimate the age of a variety of tissues. Due to the fast rate of racemization of aspartic acid, it is the most commonly used amino acid for age determination. There are various factors which affect the rate of racemization of the L-form amino acid to the D-form amino acid and these are pH, temperature, humidity, etc. As the process of racemization is a continuous one, with the formation and removal of the amino acids, the tissues which have a low metabolic rate provide a more accurate estimation of age than those which have a metabolic rate. With this bit of information in mind, the tissue of choice for this analysis is teeth. Another reason why teeth are used for this analysis is the fact that if the post-mortem interval is too long, then the only parts of the body that the forensic scientist will have left to run the analysis are the teeth or bones. The analysis of aspartic acid racemization for the sole purpose of age determination has been performed on both tooth enamel and the crown dentin. The analysis of the crown dentin has shown to generate a more accurate estimation of age over that of dental enamel. Despite positive results overall, for investigators who have repeated this analysis, some have reported that there level of accuracy has not been high and that there is the existence of inconsistencies. The other method of analysis is, radiocarbon analysis of tooth enamel (which was mention early in this section), was discovered in 2005, so is a new technique and so has yet to be thoroughly tested by the forensic community. The information provided by the technique of radiocarbon dating of enamel, is the date of birth of an individual. With this knowledge and the knowledge of the date of death (if it is known), an estimation can be made as to the age of the individual. Over the past several years the technique of radiocarbon dating of the dental enamel, has proved to have a high precision as to the determination of the date of birth of both identified and unidentified individuals. The year of the tooth formation based upon the levels of radiocarbon present in the tooth enamel is the rationale of the method. The radiocarbon (or carbon-14 as it is also known) is naturally produced within the atmosphere by cosmic ray interactions with nitrogen-14. The atmospheric concentrations of the radiocarbon have been reflected within the isotopic carbon content of new plant growth. The formation of the enamel can occur over a period of several years in humans. The upper limit of the formation of enamel has been used for the radiocarbon analysis of the age of the tooth; as this balances out the lag periods of the incorporation of carbon-14 from the atmosphere to the body. An example of where these techniques were used is as follow:

In a homicide case in Sweden, a victim's identity was unknown. During this case they illustrated how the techniques mentioned above could be combined to provide important information as to the year of birth of the deceased, the estimated age of the person at the time of their death and then to establish the date of their death. These techniques are a particularly valuable forensic tool for the police authorities as they can be used to define a group of possible matches within the early stages of the investigation they are under taking. On the occasions when the date of death cannot be established, the analysis by radiocarbon can be used in conjunction with the analysis of aspartic acid racemization of the tooth dentin so that a year of death and an age of the victim could be determined.

The experimental procedure is as follows:

Forty-four teeth were collected from dental clinics within Sweden, with consent from all of the patients willing to participate. A tooth number, date of extraction, date of birth (the year and month) and the gender of each participant was recorded. Four teeth that belonged to an unsolved homicide case were received by the Swedish police authorities; in this case, the date of birth and the date of death were not known to the analyses. In the cases where the aspartic acid racemization analysis of dentin and radiocarbon analysis of the enamel were performed upon the same tooth, half of the whole tooth and the complete root were used for the aspartic acid racemization step of the analysis and the remainder of

the crown was retained for the radiocarbon analysis. The analysis for the aspartic acid racemization was preformed according to instruction from previous work. The teeth were cut by a low speed cutter into 1-mm-thick median longitudinal sections. The other areas of the tooth except the dentin were carefully removed from each of the sections by the cutter. The dentin was rinsed with ultrasonic waves in a 0.2 molar Hydrochloric acid, rinsed in distilled water (three times), rinsed in ethanol, and finally in ethyl ether for approximately 5 minutes. The sections of dentin were pulverized by using an agate mortar and 10mg of the powder taken to be used as a specimen for the determination of the racemization ratio. The L- and D- asp were both measured by gas chromatography using a glass capillary after hydrolysis and derivation.

The average at which the enamel formation is completed for each specific tooth has been determined previously and is dependent upon the tooth number and the person gender. The average time for enamel completion for male and females is calculated, if the sex of a person is unknown. A curve graph of the atmospheric carbon-14 is plotted with the measurement of the carbon-14 concentration within the tooth enamel against the time of determination of the year of the enamel synthesis and the date of birth of the individual. (Alkass et al, 2010)

1.2.6 Amino Acid Racemization Dating Method

A new method of dating which is based upon the rate of racemization of amino acids within fossil materials was announced as a discovery by Jeffery Bada of the institute of oceanography at a conference in august 1972, which was widely published (Bada, 1972). He was quoted saying that he had discovered a basis for the method in 1968, which was so obvious and simple he was amazed that it had not been earlier. (Duane. 2000).

In the series of papers that were published by Hare et al 1969, showed that this method had been discovered and recorded.

Within proteins, there can be found sub-units or 'building blocks' know as amino acids. Within each protein there are around 20 different kinds of amino acid. Chemical bonds can be formed between individual amino acids, as each amino acid consists of an amino group and carboxyl group. A chain of amino acid can be extended indefinitely as the amino acid one can join the carboxyl group of amino acid two so that a peptide bond is formed. The carboxyl group of amino acid two can join to the amino group of amino acid three and so on. A long peptide chain is formed as the amino acid link together. Within a single peptide chain, there can be around fifty (50) several hundred amino acids present. (Duane. 2000)

VI. RESEARCH METHODOLOGY

The research methodology used for this work was based on practical experiment on the synthesis of dipeptides. The particular method chosen relates exactly to the objectives and aims of this research. Hence the use of the experiment to show the synthesis of dipeptides, and also to test the hypothesis "application of dipeptides in forensic science"

VII. PROCESSES INVOLVED IN THE SYNTHESIS OF DIPEPTIDES

Before the experiment was commenced, the weight of the round bottom flask was measured and found to be 166.86g. Once the round bottom flask was weighed, an ice bath was prepared by putting ice into a plastic container, with a small amount of water and by adding a large quantity of salt (Sodium Chloride). Within the salt bath a 100ml beaker was placed in the middle of it. In this beaker 50ml of Methanol were added and allowed to cool. In order to determine the quantity/volume of thinly chloride to add, the following calculations were done:

5g of Phenylalanine $N=\frac{m(g)}{Mr}$ $N=\frac{5}{84}=0.030m$

Mr=84

The ratio of Phenylalanine to Thionly Chloride is 1:1.1 so the number of moles of Thionyl Chloride = 0.030 * 1.1 = 0.033M.

The density of Thionyl Chloride = 1.64g

The mass of Thionyl Chloride $=\frac{0.033}{119}$ = 3.96 ml/g

One of the problems which occurred with this part of the experiment was with the measuring of each of the compounds used as they had to be weighed within 0.001 of a gram and it was difficult not to be a microgram over or under the required weight. The flask which contained the compounds was not allowed to get above a temperature of 40°C. A

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magnetic stirrer was placed within the beaker and placed upon a magnetic stirrer for a minimum period of 12 hours (was stirring for 18 hours).

The stirrer was removed with a magnetic and washed down with methanol to remove whatever of the compound was upon it. The round bottom flask was placed upon a rotatory evaporator until the sample was completely dry. The dry sample which was produced came in the form of a white solid. The round bottom flask was then weighed so that the yield of the crude product could be calculated. The weight of the flask after the solid was formed was 176.29g. This value taken away from the initial weight of 166.86g gave a yield of 9.45g for the crude product. Recrystallization of the compound was completed by adding a minute amount of hot methanol so that the compound just dissolved, followed by the addition of a minute amount of ether so that the dissolved compound, just turned cloudy. The contents of the flask where then left to cool. Once cool, the flask was placed upon a hot bath so that the methanol within the flask could be evaporated off, leaving the compound crystals.

The product was washed with a few drops of either (just enough so that the product was just wet) while under reflux. This washing process was repeated twice. The watch glass which the product was to be placed upon was weighed for an initial value which was 39.33g. Once the product was washed it was placed upon a watch glass and placed within an oven at a temperature between 75°-80°C so that it could completely dry out. The product was within the oven for a period of 5 hours. After the product had completely dried the watch glass was weighed again do that a yield of the pure product could be determined. The weight of the watch glass with the sample was 41.76g; this gave a yield of 2.43g of pure product.

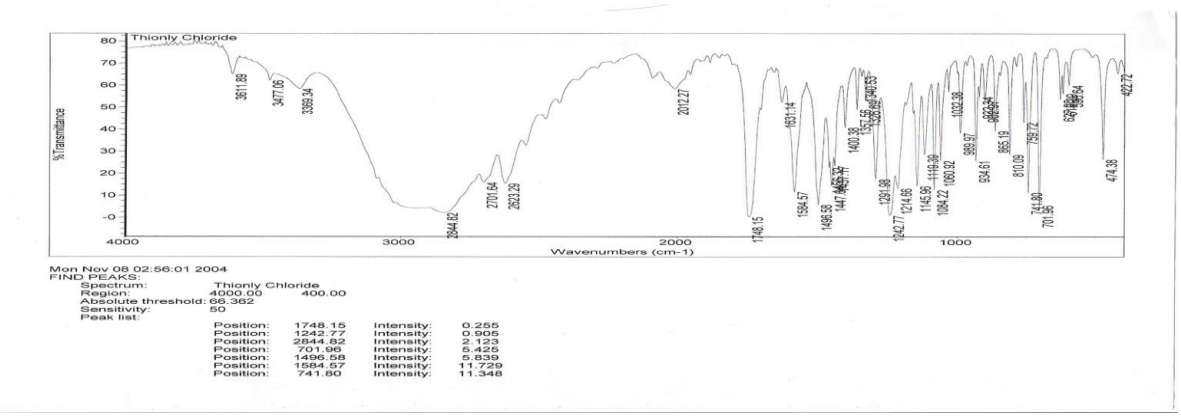
In order to get an IR of the final product, a KBr had to be constructed. To construct this KBr disc, 0.2g of KBr was weighed out and 0.002g of the product was weighed out and placed within a pestle and mortar and crushed together. The crushed compound was then placed within a KBr pellet press for 2-3 minutes at 7 tons of pressure, resulting in a 13ml disc, which was placed within an IR spectrometer and a spectra obtained which can be seen as appendix 1. Melting points of the product were also obtained by placing a minute amount of the product within a melting point tube and placing this tub within a melting point apparatus. This process was repeated five (5) times at two (2) different settings. The table below indicates the melting points obtained.

Table 1: A table showing the melting points of product.

Serial Number	Melting Point	Setting
First	136°C	8
Second	138°C	7
Third	142°C	7
Fourth	144°C	7
Fifth	142°C	7

From a research of different literature it was concluded that the melting point range for L-phenylalanine Methyl Ester Hydrochloride was 158-162°C.

Spectra obtained from the IR spectroscopy



Graph 1: IR spectra of product

The IR graph above was compared with an IR graph from a past experiment to confirm that L-phenylalanine Methyl Ester Hydrochloride had been made. (Appendix 1 shows the original IR graph obtained and Appendix 2 shows an IR graph from the literature.

Table 2: Table of groups present in molecule and their respective wave numbers

Group	Wave number (cm^{-1})
aromatic C═C	1500 cm^{-1}
aromatic C═C	1580 cm^{-1}
esters and lactones	1735 cm^{-1}
ammonium ions	2400–3200 cm^{-1}
carboxylic acids	1250–1300 cm^{-1}

The significance of table 2 (Table of groups present in molecule and their respective wavenumbers) above is to indicate the specific functional groups within the molecule obtained so that an appropriate daigram can be draw to show the shape of the molecule and its dimensions.

Table of Melting points obtained

Table 3: Table showing the melting points of product at different settings

Serial Number	Melting Point	Setting
First	136°C	8
Second	138°C	7
Third	142°C	7
Fourth	144°C	7
Fifth	142°C	7

VIII. DISCUSSION

As bones are resistant to environmental influences it makes them particularly valuable in the forensic setting. When they are intact for a long period of time, they can provide a possible identification, even of very old skeletons (only if the ante-mortem data is available). As there is no radiocarbon exchange in the enamel of a mature during a person's life time and there is usually non after death, the mature teeth are particularly suitable for radiocarbon analysis. There are strengths and limitations to both of the techniques mentioned above. One of the strengths of the radiocarbon birth dating method is that it can tell the date of birth of a person regardless of their time of death. One of the limitations of the radiocarbon birth dating method is that the window of time for the analysis is very limited to subjects who were born after the early 1940s, as all the calculations are based upon the measurement of bomb pulse-derived carbon-14. The aspartic acid racemization analysis of dentin gives us information about the age of an individual at the time of their death. This can be accomplished as the chemical conversion of L-enantiomer to the D-enantiomer will completely stop after death. This indicates that the analysis is independent of the calendar year of the person's birth and death.

IX. CONCLUSION

Within fossil bone samples of a known age, the L- and D-amino acid composition was determined. The radiocarbon method was used to determine the age. With the complimentary technique of aspartic acid racemization within dentin of teeth, it was possible to determine an individual's, date of birth and there age if the date of death was known. These techniques have been, not only a success in the area of Forensic Science, but they have also been a crucial part of the analysis conducted for certain investigations. Without these methods some investigations, into an individual's identity, would still be ongoing. The only limitations to these techniques were the temperature, the pH and the possibility of contamination occurring.

One of the most challenging aspects of this work was to find appropriate material which could be used to illustrate the different techniques that could be used for the age determination of a skeleton, which could have come from a scene.

These difficulties were overcome by conducting my searches with the following key words: amino racemization, age determination and dipeptide synthesis.

I cannot conclusively say how the experiment that I conducted relates to the literature, as I only conducted one step within the overall experiment. With further work within the laboratory to complete the next series of steps, I would be able to determine how they directly relate to the literature that I have read.

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