ARCHMAT

(ERASMUS MUNDUS MASTER IN ARCHaeological MATerials Science)

Mestrado 2 Ciclo

Exploring Trace Elemental Analysis of human remains from San Pablo Medieval site using ICP-MS

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Resumo

A abordagem analítica do uso de Trace Elements (TEs) pode ser usada não só para entender o ambiente vivo e a dieta, mas até a diagênese pós mortem de qualquer resíduo humano usando a variabilidade nas composições elementares de dentes e ossos. Oito indivíduos foram amostrados do convento de San Pablo em Burgos usando seus dois tipos de tecido ósseo (ossos cortical e trabecular) do fêmur e esmalte dentário para cada caso. Três dessas amostras pertenciam à nave da igreja (século 16^{th} -19^{th}) enquanto o resto era encontrado no pátio do claustro pertencente ao século XIV-XVI. Essas amostras foram processadas na Espectrometria de Massa Plasmática Acoplada Induzamente (ICP-MS) para analisar as concentrações de Ca e P, bem como os TEs não essenciais e bio-essenciais, a fim de poder estabelecer a integridade das amostras e para descobrir quais TEs podem ser úteis para fazer inferências sobre a dieta antiga e a absorção diagenética e em que medida. Os dados de TE foram tratados usando diferentes ferramentas estatísticas e testes para encontrar possíveis diferenças de gênero ou mesmo diferenças intra-locais na dieta e também foram corroborados com informações coletadas a partir da análise de *microwear*. Verificou-se que a dieta dos indivíduos era de tipo misto com componentes vegetais e de carne, enquanto a presença de alimentos marinhos não pôde ser confirmada. Isto foi de acordo com os resultados de *microwear* para algumas das amostras. Usando as razões de Ba e Sr por Ca, verificou-se que os ossos corticais deram os resultados mais confiáveis para inferências sobre dieta, excluindo o uso de níveis de Mn e Fe que foram altamente afetados pela absorção diagenetica nos tecidos ósseos. Além disso, as amostras enterradas na nave da igreja podem estar consumindo mais proteínas de carne do que as enterradas no claustro, o que pode indicar uma diferença em seu status social ou uma mudança na dieta ao longo do tempo. Como esperado, para a maioria dos elementos, como Pb, Mn, Fe, Cu, os tecidos trabeculares foram os mais afetados pela absorção diagenetica, ademais, na em sua maioria superfície interna do eixo do fêmur.

Palavras-chave: Trace Elements, ICP-MS, San Pablo, palaeodiet, diagénese, microwear, estatísticas

Abstract

The analytical approach of using Trace Elements (TEs) can be used not only to understand the living environement and diet but even post-mortem diagenesis of any human remains using the variability in the elemental compositions of both teeth and bones. Eight individuals were sampled from the convent of San Pablo in Burgos using their two types of bone tissues (cortical and trabecular bones) from the femur and tooth enamel for each case. Three of these samples belonged to the church nave (16^{th} -19^{th} C) while the rest were found from the cloister courtyard belonging to 14^{th} -16^{th} Century. These samples were processed in solution mode Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to analyse concentrations of Ca and P as well as both non-essential and bio-essential TEs in order to be able to establish the integrity of the samples and to find out which TEs can be helpful in making inferences on ancient diet and diagenetic uptake and to which extent. The TE data was treated using different statistical tools and tests to find possible gender differences or even intra-site differences in the diet and was also corroborated with information gathered from microwear analysis. It was found that the diet of the individuals was of a mixed type with both vegetal and meat components while presence of marine food could not be confirmed. This was in accordance with the microwear results for some of the samples. Using the Ba and Sr ratios to Ca, it was found that cortical bones gave the most reliable results for inferences on diet excluding the use of Mn and Fe levels which were both highly affected by diagenetic uptake in the bone tissues. Additionally, the samples buried in church nave might be consuming more meat proteins than those buried in the cloister which might indicate a difference in their social status or a change in the diet through time. As expected, for most of the elements such as Pb, Mn, Fe, Cu, trabecular tissues were the most affected by diagenetic uptake moreover mosly at the inner surface of the femur shaft.

Keywords: Trace Elements, ICP-MS, San Pablo, palaeodiet, diagenesis, microwear, statistics

Acknowledgments

First of all I wish to be grateful to the Universidad de Burgos and especially my supervisor and co-supervisors for guiding me throughout the length of this research. A special mention of the Parque Scientifico Technologico (PCT) lab and my labmates is necessary who worked with me as hard as possible all the way until the end. Without such assistance, this work might not have been produced. There was never a time when they were not available with their guidance and support. I cannot thank enough my friends, classmates and relatives for giving me the motivation to keep going even when the going seemed tough at times. I am deeply grateful to my friend and classmate, Rebecca MacRoberts for her timely support and guidance and also to Pedro Barrulas for taking time from his busy schedule to help with the thesis. And lastly but not the least, the ArchMat program and everybody associated with it, for giving us such pathbreaking opportunities in the course of two years. Surely, it has been a life changing experience for all of us involved.

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1
Introduction

The convent of San Pablo de Burgos was one of the first and most important monasteries that the Dominicans founded in Castile, Spain. Many artists took part throughout its construction across the centuries, who made the cathedral famous and some of them later even asked to be buried in one of the chapels of the church. It was located in the city of Burgos founded by Santo Domingo of Guzman in the year 1218 (Conde et al., 1982) according to tradition but most of the historians place its foundation in the year 1224 (Serrano, 1997). It is unanimously agreed upon that the Dominican friars were already established in Burgos between 1219 and 1222.

The historic city of Burgos was the capital of Crown of Castile at one point of time and could be considered as one of the most important centres of medieval Spain. In that time, the convent was an important landmark of the urban Burgales landscape. The history of the convent is in a way entwined with the history of this city. If its history, very close to that of the city, was substancial in historical times until the 18th century, its ruin in the 19th century was also absolute. The oldest site of the convent was located outside the city which was later shifted to its last location. Today, after having gone through turbulent times such as Spanish War of Independence and Spanish confiscation in the nineteenth century, the convent ceases to exist not only physically but also from the citizens' memory who do not even remember the existence of the convent. The monastery undoubtedly has a lot of archaeological, historical and artistic information about the thriving middle ages of Burgos city and the surrounding areas which have been continuously inhabited since more than 800,000 years ago. The life of the San Pablo convent had always been in close relation with the life of the city and needs to be studied and thus recued from oblivion in order to return it to the popular culture and memory of the city.

Being one of the most influential religious institutions in Burgos city during the 13th to 18th centuries, the convent was preferred by the contemporary inhabitants as the burial place regardless of their economic or social status. Unsurprisingly, numerous burials were recorded in the site during the excavation in 2002-2003. The convent was developed into a prison, barracks and even hospital during the second half of 19th century. By 1870, the ministry of war ordered

the still standing ruins of the convent and the church to be demolished for building barracks. Consequently, the convent completely disappeared and the barracks continued to be used for one more century until they were destructed in 1973. By 2010, the museum of Human Evolution was developed on the same site but not before a thorough archaeological investigation during 2001-2004.

The historical, archaeological and anthropological studies on the remains from San Pablo have already provided a lot of information about the medieval communities and their lifestyle in the contemporary times. Nonetheless, there have not been any previous archaeometric investigations into the remains from San Pablo whose history could be recorded better by understanding various aspects of the lost site. The main research goals of this study are to explore the diet of some of the individuals buried in the cloister courtyard and the church nave of the convent in order to find differences based on genders, socio-economic status or chronological period of these burials using trace elemental analysis and statistical tools such as t-tests, regression analysis, ANOVA, cluster analysis and a few others. Apart from this, it is also a focus of this study to be able to make preliminary explorations into developing a methodology to notice diagenetic changes in different TEs using different skeletal elements from the same specimen which might be helpful in cases like this where the original archaeological context and the soil from the site is not available for further examinations. It will be interesting to note which TEs and which skeletal tissues (tooth enamel, cortical bone or trabecular bone) provide more reliable data for inferences on diet and diagenetic uptake.

2
Historical and Archaeological Context

Burgos is located in the Castile and Leon province of Spain which has been the most important region and even an important centre of Spain in the past. The city is surrounded by Miocene formations consisting of limestones along with marls while the actual city was fed by seven rivers in the past. Thus, it is situated on river terraces most important of which is the Arlanzon River. These are therefore quaternary fluvial sediments made up of gravel, sand and clay. The monastery most probably was situated on terraces created by Arlanzon, Urbel, Ubierna and Vena rivers (Figure 1).

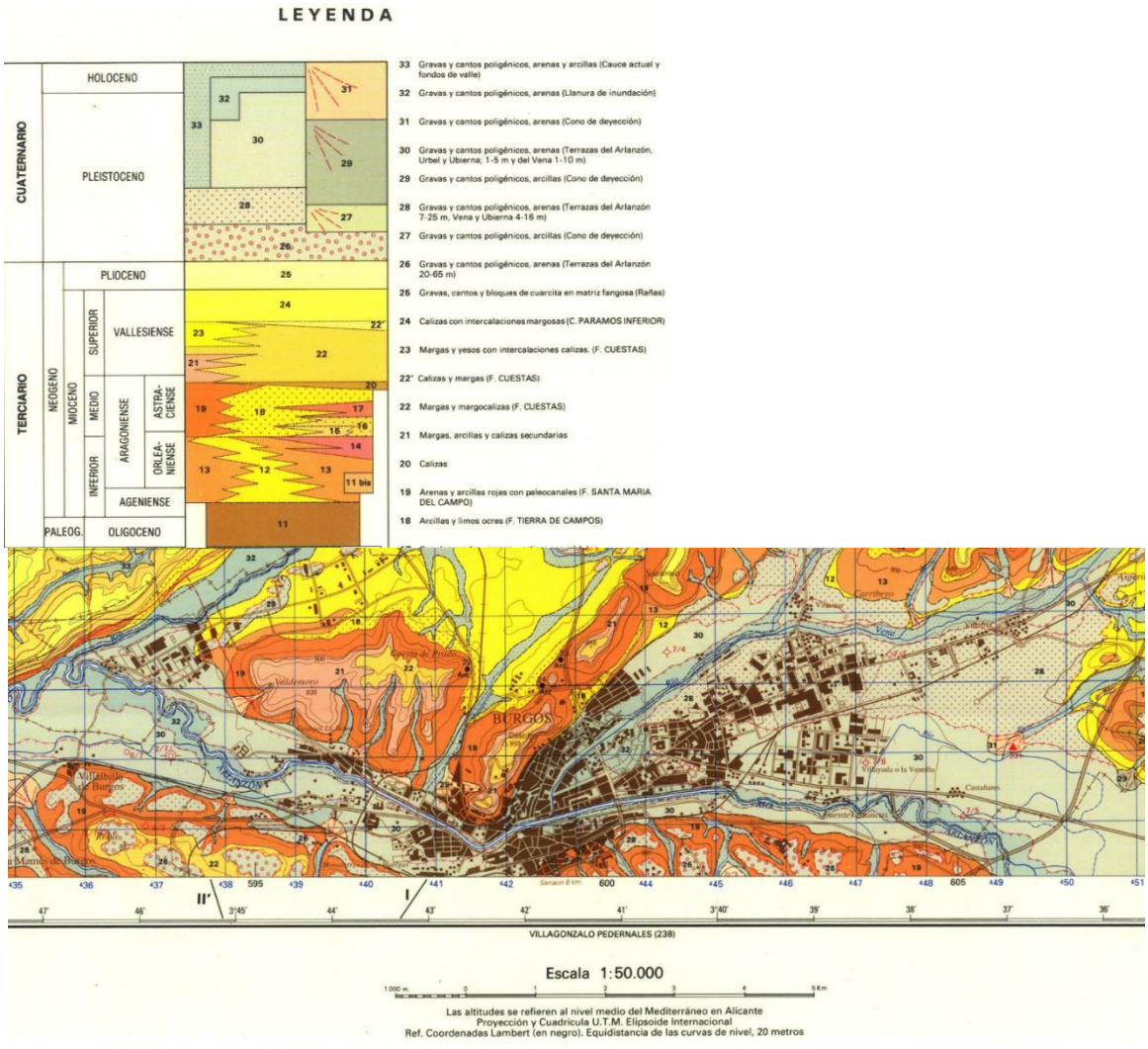


Figure 1: Geological map of Burgos along with the legend (info.igme.es)

The convent of San Pablo was the point of reference in the urban Burgales landscape and was a Dominican establishment. Although it was difficult to know exactly where the primary location was and also the exact date of its foundation. The excavations have established the location of its last standing claustro at the coordinates of 42.339073°, -3.697135° (Figure 2).

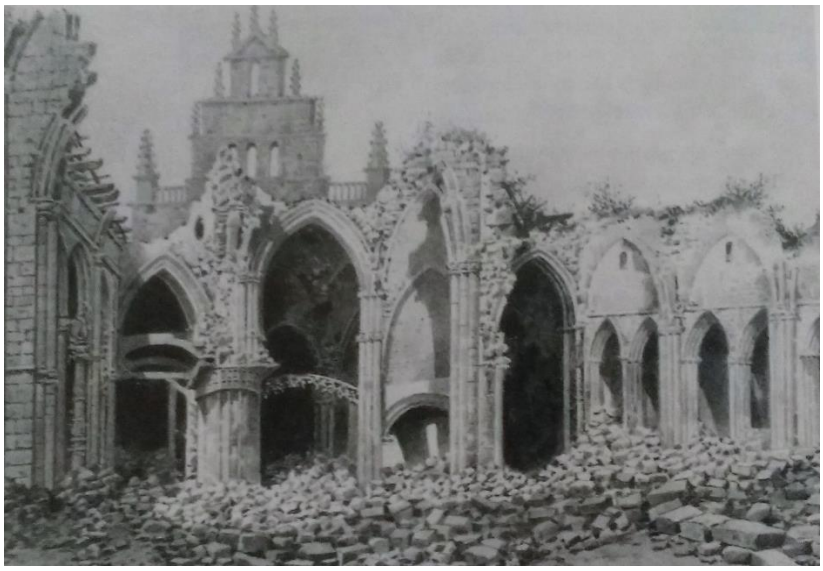


Figure 2: The view of the San Pablo monastery from Isidro Gill (Casillas & Alvarez, 2005)

We knew in 1218, that when the Santo Domingo of Guzman visited Spain on the 22 of December of 1216, honorio 3rd approved the order of preachers in Burgos (Casillas, 2003). It has also been determined that the first Dominicans settled in the city of Burgos around the 1220. They settled in the extramural of the city in the famed neighborhood of La Vega, close to the churches of Saint Cosme and Saint Damian in the south of the city (Casillas, 2003). Right from its foundation, the convent reveled in the patronage of the kings of Castile until the rule of Catholic Monarchs.

The Bull of Vitute Conspicuos established the Dominican friars as independent from the diocesan friars and tried to eliminate the ambiguities regarding the burials in the temples and monasteries, giving full rights to the Dominican friars to decide about the burials. Nevertheless, the cathedral of Burgos held a long argument with them refusing the burials of clergy and nobility members inside the convent of San Pablo (Casillas, 2002). The clash started when they denied the burial of Juan Tomé in the convent (Casillas, 2003).

This resulted in a lawsuit filed by the Dominican friars at Rome drawing until 1302 when at last the convent and the Burgos cathedral made a settlement in which the Dominicans were to move

from their convent to a new building which is the site of the Museum of Human Evolution in Burgos today (Casillas, 2002). By the beginning of the 14th Century, the Dominicans had moved to their new monastery and it took them about 15 months to transfer their possessions and the bodies buried in the old building to the new convent (Serna, 1945). The real estate and monetary assets of many convents in this area including San Pablo increased after the 1470s due to the joining of many new individuals in the convents which led to their expansion and increase in the influence (Ocampo, 2009). The main benefactors who helped the building of this convent were the kings Don Alfonso el Sabio and Don Sancho the IV, his son, who gave a place to build the new convent.

The fields that the community had occupied were close to 27000 sq metres. In the street of San Pablo there were different buildings that, after the confiscation of 1835, were sold like separate buildings. The convent that bordered these fields, had a series of properties attached to it like an orchard or an estate of recreation that was in La Quinta. And it also had a separate entrance called the noble door that was in a flank of the façade of the church and a second door for the service at the back adjoining with the San Lukas street (Figure 3) (Casillas, 2003).

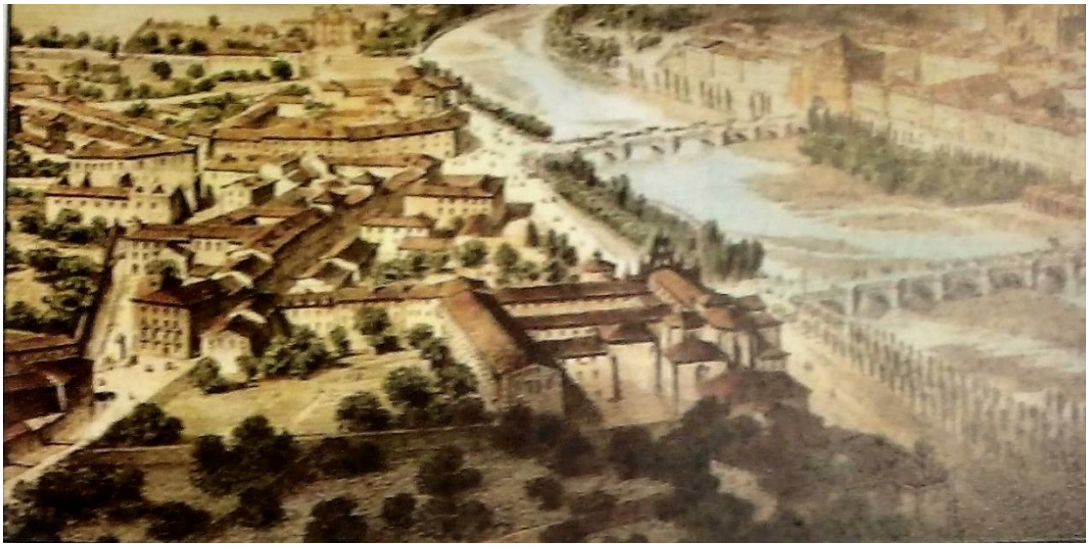


Figure 3: Engraving of the city of Burgos, of century XIX, with the view of the convent from the east, by Guesden (Casillas & Alvarez, 2005)

The 13th century is the period in which the order acquired its moment of expansion. This was an order that soon earned the appreciation of the city, thanks to its preaching and cultural advice. They were preferred in matters of devotions and especially in matters related to death, and were solicited as business advisors and contractors, as witnesses and as testamentary agents. At the end of the 12th century, and at the beginning of the following, it was the time period when the mendicant orders in Castile had a privileged position and when there were marked relations between the order and the Castilian oligarchies. This is reflected by the beginning of sponsorship of the chaplaincies, where they began to be buried (Casillas, 2003).

The 14th century is marked by the entrance of the Black Death in Spain (1346) which struck Castile hard. As a result of this, the power of the convents began to wane and the relaxation of the clergy was encouraged. With the intention of recovering the gaps in the order and the past and the plague, they began to deliver habits with great ease during this relaxation time which is known as the "claustra" (Casillas, 2003). Even with these internal weaknesses, the patronage and the burying of the dead continued to increase.

The convent maintained the continuum, but with little real support. So it could carry out some constructive activities among which the most innovative is the one that highlights the patronage of D. Leonor Henriquez, the granddaughter of Alfonso XI, who had a bulky sepulchre built in the center known as the tomb of the "beata" (Casillas, 2003). The 15th century was a good time for the convent under Bishop D. Pablo de Santa Maria, of Jewish origin, who had converted at maturity and was the bishop of Cartagena. He took possession to govern the diocese of Burgos in 1425.

His works were outstanding and promoted the improvement of the customs and important works in the diocese. He showed his distinguished patronage to the convent of St. Paul by choosing the Great Chapel for his burial as well as for those of his most notorious descendants. Among the works he performed as patrons were the finishing of the main chapel, covering of the vaults of the church, widening of the chapel of the chapter and other works in the cloister. This is the time when the convent acquired the form that it maintained, with small modifications, until its disappearance. The convent was not finished until September 31, 1430, having begun work 130 years earlier and marking the arrival date of the Dominicans around 1220 (Casillas, 2003).

The period between the end of the 15th century and the first half of the 16th century was the best time for the convent, which became an important study center for the formation of members of the

Order and for the education of the most notable people in Burgos. On the subject of death, the Dominicans were the favorites after the Franciscans and the convent was for many Burgaleses, the place chosen for their eternal rest. So the 16th century is marked by the greatest burial movement in the convent (Casillas, 2003).

The best time during the century was when the mercantile bourgeoisie appeared. A bourgeoisie whose development contributed to the best time of the city, which affirmed and consolidated its influence in the kingdom and materialized the creation of private chapels within the convent of San Pablo. As far as construction was concerned, by the end of the previous century the four wings of the cloister had already been closed, and other offices such as the library, dormitories, refectory, nursing, hostelry and novitiate were expanded.

By the second half of the 16th century the plague returned to the city. This was the main cause of Burgos's loss of demographic, economic and court power resulting in the fall of the Convent of San Pablo, which sent its best men to the convents of Valladolid or Salamanca. The decadence is also accused in terms of patronage having declined to its lowest intellectual level. The works that were carried out at this moment were no longer inside the convent edifice but in the premises of the convent with too much repetition and with an apparent lack of necessity, for example, structures like the prestiberio, the bookshop, the stairs or the room of "De Profundis" were modified several times in the same century (Casillas, 2003).

In the second half of the seventeenth century the last works of importance in the convent were carried out. Different chapels and facade of the building were modified, building a sumptuous belfry under the orders of Friar Jose de Torres, in addition to the rebuilding of the cloisters and of their ornamentation with pictures (Casillas, 2003).

But without a doubt, the booming years for the convent of San Pablo ended in 1807 when the Napoleonic troops arrived in Burgos, at which time the city had to establish barracks, schools, and private houses and evidently also used the convents for these military purposes. The troops occupied the entire ground floor, except for the inn and the kitchen, an occupation that lasted until 1808 when, after the Battle of Gamonal having defeated the army from Extremadura, the troops entered the city under the consent of General Lasalle, setting it on fire and plundering it for days (Casillas, 2003). The building suffered by ruination, the church was dismantled and was without altars.

During the period of military occupation the convent was destined to diverse uses, like lodging of troops, jail for the prisoners, military hospital and even warehouse. Several friars were executed and the rest of them left the convent. Their property was plundered and destroyed, some were saved, transferring them to numerous parish churches, such as San Lesmes, San Gil or the Cathedral, as well as to several towns in the province.

After the departure of the weak troops of the city, some of the friars returned to the convent in 1813, the Friar Tomas de la Iglesia being the head. They rehabilitated the rooms and according to the chronicles of that time, it was remarkable how quickly the convent returned to its day to day functioning (Casillas, 2003).

In 1827, thanks to the Friar Manuel Martínez, the works took great impulse but the joy lasted for a very short time, since in December of 1835 the friars were expelled from their monastic dependencies. After the expulsion, the objects of worship and the estates began to be sold. On the other hand, the buildings, the church and the convent were occupied by the army, for the lodging of troops, hospital or ammunition store. Thus it deteriorated, until the army itself decided to destroy it in order to build the barracks that were inaugurated on June 19, 1883 and remained standing until 1975 (Casillas, 2003). Few monasteries achieved such a restoration and a few have known such a disastrous end after the exclaustration of 1835. The engravings from the ruins kept in the City Hall show us that it was a sumptuous building and the church especially was an architectural marvel.

2.1 Structure and architectural insights

It can be mentioned that there is no proper Dominican architecture. In the beginning, the order of the Dominicans felt a great disinterest in building their edifices. But things changed from the XIII century when the order of the mendicants proposed a new typology that had a great acceptance in Spain, a same aesthetic taste that the Franciscans as well as the Dominicans shared. This type of buildings were raised close to the local tradition, seeking to maintain the functionality. Buildings of that time were being gradually modified to adapt to the fashion of the moment.

In the first attempt, the friars looked for a space where they could officiate the Eucharist, and later extended the wish to have a great choir, which influenced the design and a polygonal form was developed. This was united with a body formed by one or three naves and generally a roof of wood.

The ornamentation at first was very scarce but to cover the pedagogical needs the Cistercian rigidity was abandoned and they began to open up to the religious arts. Until the end of the 14th century it was not possible to even mention the entrance of images in the Dominican temples, although in Burgos in the second half of the 14th century, the cloister was decorated pictorially (Casillas, 2003).

An artistic austerity existed in the primary years which has nothing to do with what happened in the future. The changes in altarpieces, great artistic works and numerous paintings covering the walls of the monastic stays account for the works from great artists of the time.

The construction stages for the convent can be divided into three specific periods (Casillas, 2003).

a) A first period that covered the 14th century and reached the beginning of the 15th century, where the basic plan was built.

b) A second phase, in the first half of the 16th century, in which the convent was widened and decorated in the plateresque style.

c) A third Baroque period, which was going to be maintained until the beginning of the 19th century.

The large church was attached to the convent on the north side with a structural plan of a Latin cross and nave, besides having a greater chapel. The convent of the two floors was organized around a large cloister, the lower part with a more public character and the upper part dedicated to the needs of the community. Each wing of the cloister was also dedicated to a function, the north wing being for the passage, the east wing was the noble zone with the chapter room, the wing of medidodia was destined to the life of the community and the west wing was dedicated to the study (Casillas & Alvarez, 2005). The materials used were those being used commonly in the city of Burgos such as Hontoria's stone (a pure limestone), brick, wood, plaster and stucco.

2.2 The archaeological background

The first action carried out on the remains of the convent of San Pablo (Burgos) was carried out in 2001 (February-April), under the orders of the archaeologist Jose Luis Ibarra, by the company Wyngaerde. This intervention had the objective to verify the existence of material remains of the

Dominican convent, to be able to define in detail the construction phases of the convent. In addition to the archaeologists mentioned, the team included researchers José Miguel Carretero Díaz, researcher Rolf M. Quamm, and paleontologist Yahya Bensaid who conducted a study on the animals consumed in the monastery, to provide data on daily life and diet of the friars (Casillas & Alvarez, 2005).

Prior to the archaeological intervention, a survey was carried out, in which it was possible to verify the existence of walls, ceramic remains and human remains, as well as a pavement sample, which could be recovered and is currently represented in one of the buildings that make up the complex of the human evolution museum.

After the pre-excavation survey, the archaeological action was allowed, which was carried out delimiting the enclosure in different zones.The first one was called zone B, located geographically in the northwest area of the plot with dimensions of approximately 8,100 m². The surveys on the other zone (Zone C) are about 16,500 m², and were subdivided into two other zones (C1 and C2) (Casillas & Alvarez, 2005) (Figure 4).

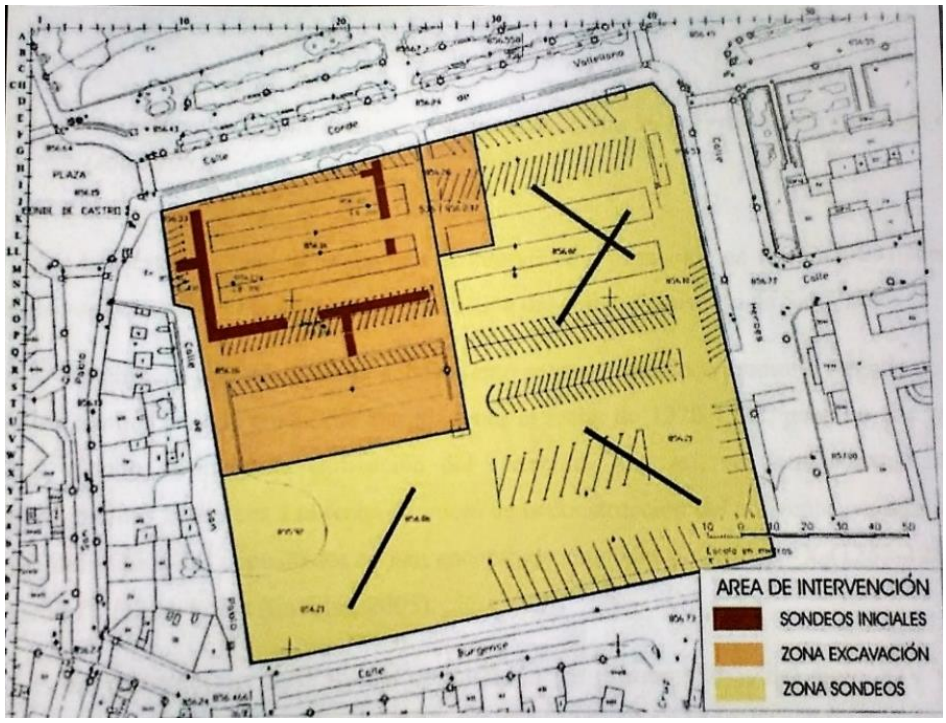


Figure 4: Map of the 2002-2003 archaeological excavation which shows the total area (22,000 m2) (Memoirs of archaeological intervention 2002-2003)

The archaeological work was decided to be started in the area of the cloister, which presented better results and remains than the remaining of the church, which was estimated beforehand thanks to the pre-excavation survey. These burials were documented through "Burial Files" in which the layout of the skeleton, the burial form, the treasure accompanying it and the stratigraphic relationships were recorded (Casillas & Alvarez, 2005).

In the church the plan of the convent was defined by obtaining the outline of the head chapel and of other chapels. In addition to this, possible exhumation of 242 burials was found in the nave and in the lateral chapels. The excavation was completed by another company "Aratikos Arqueólogos", which under the direction of Angel Palomino and Javier Abarquero, carried out the investigation between June and September 2004 (Casillas & Alvarez, 2005).

2.2.1 Evaluation of the results from the archaeological intervention (2002-2003)

The studies carried out during the archaeological intervention (2002-2003) were decisive to know the constructive sequence of the old convent of San Pablo. Referring to the history of the convent it was possible to establish how the arrival of the Dominican friars to the city of Burgos had the date of 1220-1222, but it was not until 1302 when the construction of the convent began. However, burials are recorded prior to the commencement of the construction of the convent, since along with the individuals buried there are coins of Alfonso X (1252-1284) and Sancho IV (1284-1295) (Casillas & Alvarez, 2005).

The construction of the church began in the early years of the 14th century and was completed thanks to the impulse of D. Pablo de Santa Maria in 1430. It was a Gothic church with three naves with several chapels located between the buttresses. This chronology has been confirmed by the archaeological intervention. After the church began the construction of the most essential units, among which the capitular room initially built with a short height, stands out.

The cloister was realized in several phases being primarily of low height. In the year 1380 the documents report the news of the construction of a new cloister which was completed at the end of 14th century. The cloister was used as a place of burial. Since the end of the 13th century some of these burials were adapted to the wall, so it has been possible to confirm the existence of tomb altars (Casillas & Alvarez, 2005).

In the 16th century new works and reforms were carried out in the premises of the convent. The cloister was remodeled along with the chapter room in which a second mortar floor was found with eleven burial pits. The construction of thirty tombs of cooked bricks for the members of the monastic community between the 17th and 18th centuries in the chapter room is verified.

An important work was produced in the church called the chapel of the eleven thousand virgins. It was demarcated as a funerary chapel in 1563 and given to the Maluenda family who built a crypt that was in use until the 18th century. In the 19th century this chapel was filled with children's burials, along with a large registry of architectural and decorative remains.

In the 16th century, the naves were multiplied in the Church's edifice for the family and individual graves, where burials were performed in lime-filled coffins and successive reutilizations took place with the generation of ossuary until at least 1782 (Casillas & Alvarez, 2005). With the creation of municipal cemeteries in the 18th and 19th centuries, burials were discontinued inside the convent building.

2.2.2 Burials and material remains found

The burials found in the excavation season of 2002-2003 were located in three areas: the first is a necropolis in the courtyard of the cloister that dates from the Medieval period, a second area with graves in the Chapter Room where the monks would have been buried belonging to the monastery and a society of greater social class and a third burial zone located inside the church, more or less near the high altar according to social class (Figure 5).

There have been found 428 burials and an indeterminate number of ossuaries (Figure 6). The intervention of 2004 numbered another hundred individuals and many more ossuaries which were in different chapels.

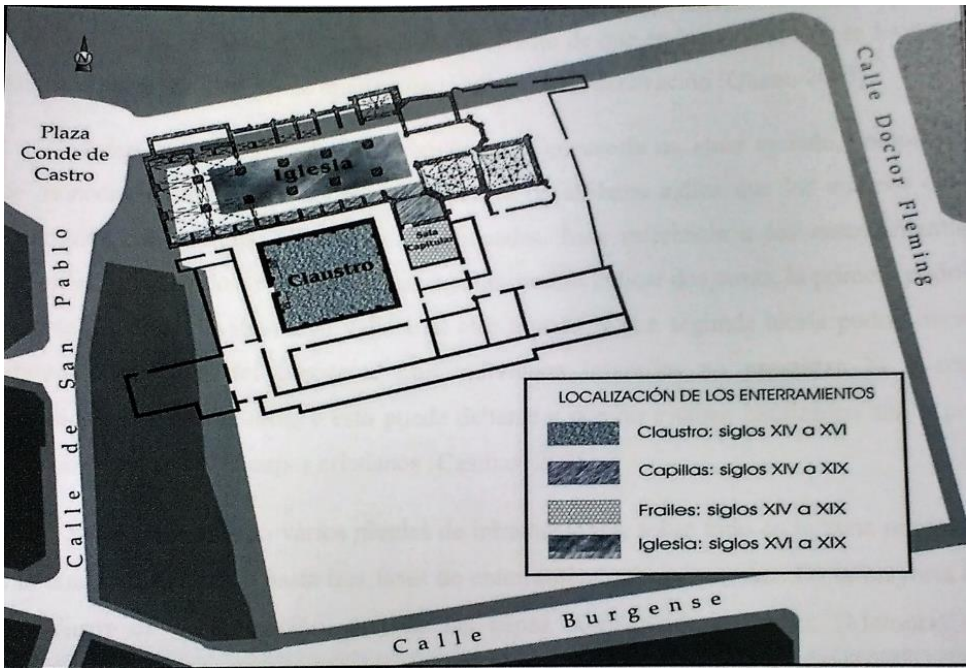


Figure 5: Map showing the location of burials (Memoir of archaeological intervention 2002-2003)

Figure 6: The burials and ossuaries found from the Convent of San Pablo (Casillas & Alvarez, 2005)

Cloister

The burials of the cloister belong to the 14th to 16th centuries, and were found in earthen graves, making a set of 118 burials of which 54 correspond to children (Casillas & Alvarez, 2005). The adult individuals buried there, appear to be oriented according to the Christian ritual that has been practiced since the Middle Ages, according to which the head lies in the west and is facing east, so that in the resurrection they stand facing god. In addition to the burials there was a varied treasure, composed of coins and copper pins. The presence of pins indicates that the bodies were buried with a shroud. Coins along with burials, due to pagan reminiscences, could indicate two things. The first that they took something valuable to the other world and a second theory could refer to the payment of the boatman. Infantile individuals did not present the same discourse as adults, and this may be because they were not yet baptized and therefore were not Christian bodies (Casillas & Alvarez, 2005).

Several levels of burials have been found, especially in the northwest area, in which up to three superimposed burial phases have appeared. In most of these cases of overlap, one of the layers corresponds to ossuary (Excavation Report, 2002-2003 (cited in Casillas & Alvarez, 2005)). The state of conservation of these skeletons is diverse, being the best conserved, adult skeletons and those located in lower levels. These burials are assigned to the stratigraphic unit (U.E) 1-250 (Excavation Report, 2002-2003 (cited in Casillas & Alvarez, 2005)).

A study of 16 individuals from this strata indicated that life in medieval times was appalling, the poverty of the diet is evident in the study of teeth and malnutrition is reflected in the growth pattern of children, well below the current one demonstrated through previous studies. The poorest population was buried in the courtyard of the cloister, leaving the church for noble characters (Casillas & Alvarez, 2005). Five of these remains will be examined in the study at hand.

Church

A second burial site is the church, where burials appear in both the central nave and the side chapels (located in the southern corridor and are total nine in number). The chronology of these burials is from the 16th to the 19th centuries. All the burials here were arranged from West to East. The state of conservation of these skeletal remains is not optimal, possibly due to a higher acidity of the substrate or due to the remodeling in the 19th century (Casillas & Alvarez, 2005)

In the 2002-2003 campaign, 224 west-east skeletons were documented (Casillas & Alvarez, 2005). There is an area of overlapping tombs at the feet of the church up to four levels. The side chapels on the other hand have a single level and a treasure rich in objects of personal adornment and coins, associated with burials. All the burials of the central nave belong to the U.E. 1-350, except for some examples of individual tomb presenting another U.E. (Excavation Report, 2002-2003 (cited in Casillas & Alvarez, 2005)). Three of the individuals buried in the central nave will be examined in this work.

Chapter Room

The capitular room is the third place of burial. It was part of the political-administrative space of the Dominican friars but it also had a sepulchral function. During the archaeological intervention carried out in the chapter hall, which was located to the right of the east part of the cloister, 26 tombs were found with their corresponding architectural structures. They were formed by varied materials: brick, stone and various moldings. (Excavation Report, 2002-2003 (cited in Casillas & Alvarez, 2005))

These burials belong to successive historical periods of the 14th and 19th centuries. An early period refers to all the burials associated with the architectural structures of the tombs. A second phase, anterior in antiquity to the first, corresponds with a second burial. A third phase, with a superior antiquity with respect to the previous ones, corresponds with a level of ossuary and under this another level of ossuary of a superior antiquity was found (Excavation Report, 2002-2003 (cited in Casillas & Alvarez, 2005)).

The intervention in the capitular room concluded with a total of 56 burials of which one belongs to a subadult individual and in most of them the head was located to the east which is the opposite to that in the cloister (Excavation Report, 2002-2003 (cited in Casillas & Alvarez, 2005)). The recovered materials are diverse such as bone buttons, remains of leather sandals, rosary beads, scapulas, coins, everything related to the rudimentary and the shroud of the friars. As a general conclusion, the excavation campaign (2002-2003) has managed to provide data on the construction of the old convent of San Pablo and confirms the historical data proposed with archaeological evidence.

3
Scientific Background

There is a vast variety in the culture and diversity among humans in both space and time. Archaeology helps to study all the various past cultures and with the advent of archaeometry it has become easier not just to understand the material remains of past humans but also their way of life, origin, mobility, health status, and diet and so on. Physical anthropology now is being assisted by the chemical and physicochemical analysis of the skeletal remains along with the conventional anatomic/anthropological study. This methodology can enhance our knowledge in various ways and can help to develop quite similar reconstructions about all the aforementioned questions and many more (Szostek et al., 2003).

Trace element (TE) analysis is a versatile analytical approach for archaeometry which can be utilized to provide basis for the reconstruction of the food economy, living habits, environment, and dietary habits of the ancient populations throughout the history of humans (Boscher-Barre & Trocellier, 1993; Molleson et al., 1988; Reiche et al., 1999; Brenn et al., 1999; Elliott & Grime, 1993).The processes of incorporation of trace elements in the bioapatite of the skeletal system are active right from the beginning of the life of the individual directly from its environment till after the death burial period, termed as diagenesis (Reynard & Balter, 2014).

Hence not just post-mortem diagenesis but even living habits of any remains can be inferred from the variability in the elemental compositions of both teeth and bones (Seiler et al., 1994). Thus it might be concluded that archaeological investigations related to ancient populations and their living habits and environment can be carried on with the help of such elemental markers by profiling the elements in ancient human remains (Carvalho et al., 2000).

3.1 Elements in the human body

The elements present in the human body have been divided roughly into three (or two in some cases) categories namely:

a) Major elements

The six elements namely oxygen, carbon, hydrogen, nitrogen, calcium and phosphorus which make up around 99%age of the human body are considered as major elements.

b) Minor elements

Potassium, sulfur, sodium, chlorine, and magnesium make up the most of the remaining composition of the body and are termed as minor elements.

c) Trace Elements

Elements such as iron, zinc, silicon, strontium, bromine, lead, copper, manganese, barium and many more make up less than 1%age of the body composition and are known as trace elements. Some of them are essential or have a favorable effect on the body, while some seem to be toxic or do not have any known function (https://sciencenotes.org/).

Some authors have classified major and minor elements together as major elements and the rest as trace elements or minor elements (Underwood, 1959).

3.2 The major elements

Phosphorus and Calcium

Phosphorus is an indispensable part of the apatite structure of both teeth and bones. The analysis of phosphorus present in the remains can be used as a measure of the extent of degradation and diagenesis acting on them. Ca/P ratio is usually measured for such inferences. Calcium makes up the largest percentage in bone and teeth mineral which is up to 38%age for bones and is more or less constant for all cases except when found in archaeological context with fully preserved collagen it can range between 26%age-38%age (Burton, 2008). Thus it is usually measured in order to assess the quality of the sample in most cases (Allmäe et al., 2012).

3.3 The trace elements

The TEs assimilated in the apatite of any living being can be broadly divided into two following categories.

3.3.1 Essential elements

Essential elements are those which are required by the human body for regulating various functions and thus play a very important role in our metabolism and other biochemical pathways. Their concentration has to be controlled in such a way as to fall exactly between the thresholds of toxicity and deficiency i.e. neither too much nor too less. Consequently, by measuring the concentration of such elements in the archaeological remains, it is possible to figure out any possible deficiencies

or toxicities in the past societies (Patterson et al., 1987; Rasmussen et al., 2008). Essential elements can help to determine the diet, metabolic activity and so on for the individual since they are important for the bodily functions in one or the other form (Reynard & Balter, 2014). They are also actively involved in the activities of the enzymes and proteins which are essential for bone growth (Yamaguchi et al., 1986).

Three important examples of such bioessential elements are zinc, iron and copper. All of these three play a vital part in the metabolism in human body. Their concentration in the body is governed to be lie between the toxic and deficient levels since they are so crucial for many bodily functions. Zinc is a component of more than 300 metalloproteins which function as enzymes or have other important structural properties (Cousins, 1985). Iron is well known to be present in haemoglobin which is involved in the transfer of oxygen and transport of electrons within the body. Copper is highly associated with iron in metabolic processes and pathways and facilitates the transfer of electrons through biochemical reactions. Manganese is another essential element whose high concentration in the human remains can give evidence of high ratios of plant foodstuff in the diet of the individual but it is not always the case (Allmäe et al., 2012).

3.3.2 Non-Essential elements

Non-essential elements are termed so because they do not yet have any recognized function in the human body but due to their similar properties to bioessential elements, they tend to replicate the behaviour of such elements. These are the elements which get incorporated in the apatite replacing the essential element. Hence in general, they are not part of any metabolic pathways and their biological behaviour is understood better by calculating their ratio in reference to that of the bioessential element that they mimic (Reynard & Balter, 2014). Their analysis can be helpful to rebuild the trophic chains by comparing their concentration with that of the essential element. They usually due to their similar size and chemical properties to an essential element, passively become part of biochemical processes and pathways in the biological organisms. For example alkali earth elements like Ba, Sr, Mg and others which might get accumulated in large amounts during the lifetime of an organism while others like rare Earth elements (REE) such as Hf, Th, U which get stored in the remains post-mortem and can give information regarding the tracing of the diagenetic processes and even dating of the remains (Reynard & Balter, 2014).

Strontium and barium are two important non-essential TEs which are associated with Ca and their ratios with Ca have now been used for over sixty years (Comar et al., 1957; Wasserman et al., 1957). The body undergoes the process of purification in which a healthy adult mammal reduces the ratio of Sr/Ca and Ba/Ca during the various metabolic pathways. This lowering of nonessential elements in the mammals leads to reduced ratios of Ba/Ca and Sr/Ca in tooth, body and bones as compared to that in the food when one moves up the trophic pyramid. Lead is another non-essential TE which, instead of being a vital part of nutrition for the body, is a rather harmful and toxic element for biological organisms. Lead is taken up by the body from the surroundings. The lungs or digestive tract facilitate the intake of lead in an individual (Bronner, 2008) which keeps getting built up in the bones throughout the life of the individual if and whenever the body is subjected to any source of lead pollution.

Apart from these two categories, there might also be some non-toxic elements which might get absorbed in the gastro-intestinal tract if they are needed by the body. Hence the composition of such elements is developed as a result of the balance between their intake in the diet and their metabolic requirement. Enameloblasts and osteoblasts are two more very particular types of cells which govern the precipitation of apatite crystals and in turn have a huge effect on the concentration of trace elements (Reynard & Balter, 2014).

From the point of view of palaeoecological and paleontological studies it is imperative to be able to differentiate the bio-essential elements from the non-essential elements since their behaviour and the processes are different in the body.

3.4 The Structure of Bones and Teeth

Teeth and bones are minerals consisting of a hydroxyapatite and protein matrix along with a calciumphosphate which is inorganic in nature. Interestingly enough, they both are helpful in monitoring the doses of various elements to which any human has been exposed to (Carvalho et al., 2004). Bones and teeth generally are composed of an inorganic matrix, an organic matrix and different cells. The creation of mineral tissues in both of them is being governed by these cells. Teeth and bones are both quite dynamic structures in their own but bones much more so than teeth. (McKee et al., 2005).

Bones and teeth are very similar in their compositions but with some vital differences owing to their distinct functions. The bones are composed of some amount of non-collagenous proteins, and collagen which is the main fibrous protein that makes up its organic matrix. It is the main protein that provides flexibility to tissues like tendons and ligaments but the actual rigidity required to bear greater loads comes from the inorganic mineral part which is present alongside the collagen matrix in the bones and teeth. Tooth dentin and cementum are also composed of collagen as the primary organic component but in the enamel there is no collagen present. The major mineral present in the enamel is similar to the hydroxyapatite mineral found in the inorganic matrix of bones. The nanocrystals present in bone apatite have a larger number of possibilities of substitution as a result of the various types of vacancies in the molar ratio of Ca/P which is not the case for the enamel apatite and thus is more approximate to the actual stoichiometric hydroxyapatite ratio of 1.67 (Boskey, 2007).

3.4.1 Teeth

Teeth are also composed of different tissues just like bones and can be differentiated at organ level. Teeth comprise of inorganic, organic and water fractions. Their inorganic stage consists of the unit cell $(Ca,X)_{10}(P,C)_6(O,OH)_{26}$. The microcations do not generally construct complex ion species and prefer coordination with oxygen instead (Liu et al., 2014). These cations have a small ionic radius and high charge/radius ratio and can substitute each other. The X in this chemical formula signifies an assortment of potential replacements for Ca, such as Sr, Ba and Pb (McConnell, 1973). The exact chemical formula for enamel is $(OH)_2Ca_6[(P_{5.8}C_{0.2})O_{24}](Ca_{3.1}Mg_{0.1}C_{0.5})$ which estimates the Ca/P ratio at about 2.02 (Gruner et al., 1937).

Tooth is basically made of four tissues namely enamel, dentine, cementum and dental pulp. The crown is made-up of these vital tissues and is covered in enamel at the top of the nape of the tooth which extends until the gumline. This enamel is very hard thanks to its almost completely mineral composition without the presence of any or very little protein. The crown of the tooth is visible once it get erupted (Liu et al., 2013). The dentin is the most important constituent of a tooth which is situated right under the enamel. This dentin then is separated from the surrounding jawbone by a composite material made of dentin and bone which is called cementum which along with the jawbone is connected with the tooth by a periodontal ligament surrounded by a membrane (Boskey, 2007).

The tooth is able to carry out its functions with the help of a regular supply of blood vessels and nerves into the pulp cavity inside the dentin. After mineralization, in early life, the enamel remains closed and will no more carry out notable physiological exchange of elements (Liu et al., 2013).

Human beings generally have twenty deciduous teeth in the primary stage of their life and thirty-two permanent teeth when they reach adult hood. Each type has its own time frame for calcification, growth and eruption (Table 1) (Hillson, 1996). Such differential growth of teeth in humans can therefore provide the possibility of combining multiple teeth of the same individual to create a composite time series using TEs. This combination allows the construction of longer continuous records of seasonal variations in paleo-environments or diets during the years in which the teeth mineralized (de Winter et al., 2016). Teeth are fundamentally classified into four groups viz. incisors, canines, premolars and molars.

Teeth were reported in 1930s to contain a variety of minor or trace elements (Dreal, 1936; Lowater & Murray, 1937) and thus conserve great data through a life span varying with environmental exposures (Liu et al., 2014).

Table 1: Development of permanent dentition (Simon & Stevenson, 1975).

Designation	**Calcification begins**	**Eruption**
Central incisor	3-4 months	7-8 years
Lateral incisor	10-12 months	8-9 years
Cuspid	4-5 months	11-12 years
First bicuspid	18-21 months	10-11 years
Second bicuspid	24-30 months	10-12 years
First molar	birth	6-7 years
Second molar	30-36 months	12-13 years
Third molar (wisdom)	7-9 months	17-22 years

3.4.2 Bones

Bone is a major part of the skeletal system. It is a living, dynamic structure which provides a supportive and defensive foundation for the body. It provides a repository of calcium and phosphate and functions in metabolism also. Core of the bone consists of marrow, which serves as a repository of nutrients and creates several forms of blood cells. The artificial segment is made of crystals which build up hexagonal plates that are arranged in a regular way on and parallel to the axis of the collagen fibers. Bones are made up of customarily hydroxyapatite, but they also consist of carbonate, citrate and lesser amounts of sodium, magnesium, potassium, chloride, fluoride, and a number of other elements (Tandon et al., 1997).

Bone tissue is divided into two sections: compact (cortical) bone and trabecular (spongy and porous, cancellous) bone based on the hardness, porosity and the content of soft tissue existent in them. Though not every bone can be determinedly categorized as compact or trabecular as some types are intermediate in porosity and challenging to classify. The compact bone is the rigid dense part enclosing the outer walls of all bones and is conjoint in the streak of the long bones. The trabecular bone is a pliable formation seen at the core of flat bones and at the edge of long bones. It is extremely filigree being soft and comprising mainly of bone marrow (Arnold et al., 1966).

Bone is an exemplar of a biological specimen that presents many challenges to acquire a sample for chemical scrutiny. Thus, it’s not surprising that dependable chemical composition data, especially for minor and trace elements, are few.

3.5 Trace Elements in Bones and Teeth

The concentration of different elements in the apatite mineral is linked with various factors such as their intake from water, food, metabolic pathways, respiration, and exposure to the environmental factors and also formation of some definite tissues while the individual is in the period of in utero development (Dolphin et al., 2005). There are certain physiochemical (external) parameters such as pH, salinity, temperature, soil composition etc. that govern the uptake of trace elements. This systematic occurrence is predictable (Darrah, 2009). TEs differ from one person to another and therefore can also be used for forensic objectives (Perrone et al., 2014). The metabolic reactions in the body do not have a strong influence on elements such as Sr and Ba which are thus more useful to derive palaeodietary inferences because of their ability to be directly correlated to

food habits of each individual. These elements are also incorporated in the body when they replace Ca in the hydroxyapatite crystal of the bone mineral and hence are also prone to diagenetic alterations as well (Pankowska et al., 2016).

Bones are one of the most dynamic structures in the human body and one of the hardest as well. They are dynamic since they keep getting remodelled periodically all throughout the life of the individual. This turnover of bone or its continuous replacement is called remodelling in which cycles of simultaneous formation of new bone and resorption of existing bones keep occurring. This process does not stop even when the growth of an individual might stop and thus leads to storing of trace elements even in adult skeletons (Swanston et al., 2012). Owing to the equilibrium between these two processes of resorption and formation, every year all through the life of any individual, about 10%age new bones are being formed in case of mature bones. The turnover rates for both of them are quite distinct, i.e. 4.3%age of the total mass of the Ca exchanged per year belongs to the compact tissue while 32%age of it can be attributed to the spongy tissues. What makes them really useful in archaeology is the fact that the bone structure survives death and thus can be encountered in fossil (Abbott et al., 1996) as well as archaeological records (Mulhern & Van Gerven, 1997).

Due to the process of turnover or remodelling of bones owing to cellular activities, there is a variation in the composition of bone depending on the environmental factors, health and the age of the tissues and the age of an individual. Even within the bone different localities within the trabeculae and osteons can have difference in their crystal size, chemical composition and in the mineral composition depending on the age of the tissues (Boskey, 2007).

Teeth also exhibit this variability in constitution and structure among the different components. For the mature tooth enamel there is no process of removal and re-deposition, in short there is no remodelling procedure for enamel (Boskey, 2007). Moreover, the organic part of its matrix is already eroded and additionally enamel is not made of a collagen matrix (Margolis et al., 2006). Thus the chemicals which can be used for repairing the damaged enamel by remineralization or some bacteria that release acids which can cause dental caries and cavities as a result of dissolution, are the only ways by which the composition of tooth enamel can be changed (Verdelis et al., 2007). There are many environmentally originated elements which get incorporated in the mineralised structures of the human body during its lifetime (Carvalho et al., 2000).

The amount of elements incorporated in the tooth enamel is much less than that in the bones (Eggins et al., 2003). This happens because hard tissues have different properties when it comes to absorption of minerals. While the enamel is being mineralized, entry of Ca is enhanced to the detriment of the Sr and Ba level and therefore they both are not present in enough quantities (Balter, 2004). Teeth can preserve ontogenetic data and are quite tough and impervious thanks to their highly crystalline structure. They are able to record the chronological development in the life of an individual in the form of element distribution at various degrees of mineralization. This variation in the distribution is caused by factors such as health, illness, stress and diet of an individual (Dolphin et al., 2005).

The research has delved deeper into micro and macro-elements and their investigation in skeletal remains in the last thirty years. This newly developed area of research has opened up new avenues of studies in the field of anthropology which offers various possibilities of research as has already been said such as studying diet, pathology and diseases (Glen´-Haduch et al., 1997), the social status and also the physiology of the ancient communities (Schutkowski, 1994; Schutkowski et al., 1999).

3.6 Main factors for TE uptake in bone and teeth

3.6.1 Diet

The food habits of past societies have long been a subject of enquiry. In case of populations belonging to historical times, information about the food can easily be found from historical records and remains. This information can be also complemented with archaeozoological and archaeobotanical remains and human evidences found from archaeological contexts.

Diet reconstructions have also been made possible by the analyses of chemical elements in the bones and teeth of past humans. Somewhere in the second half of the 20th century, the first attempt at trying to analyse the elements in human bones in order to understand and recreate the food habits of the ancient communities was undertaken by Gilbert (Gilbert, 1975) in 1975 and by Brown in 1973 (Brown, 1973). They consequently have been considered the forerunners of such studies and led to a rigorous wave of such researches in the 1980s. Since then, there have been many studies upon the dietary habits of past societies. Usually the study of essential elements plays an important

part in diet reconstructions. For example the presence of elements such as zinc (Zn) or copper (Cu) usually points towards the ingestion of meat in the diet (Buikstra et al., 1989).

Of late, trace element analysis has shifted its focus from palaeodiet reconstruction towards the understanding of diagenetic processes and their impact (King et al., 2011; Maurer et al., 2011) after settling on the fact that bone chemistry is quite complicated. The promise of a research field developed on the basis of the relation between trace elements in human apatite structures and particular trace element concentrations has now faded after the initial boost (Brown, 1974). Nevertheless, barium and strontium are still being used for diet reconstruction by scientists (Kamphaus, 2013).

3.6.2 Diagenesis

The composition of trace elements in teeth and bones is considered to be a good indicator of the level of the exposure of that individual to the elements present in the past environment and diet. But the complexities arise when these elements not only get incorporated in the body while alive but can also assimilate post-mortem from the archaeological burial environment of the remains (Swanston et al., 2012). Post-depositional chemical alteration or diagenesis in archaic human bone is the major problem in the use of trace element examination for dietary reconstruction. Due to its regular remodelling and recreation, bones are viewed as suitable dose monitors for some of the trace elements. But on the other hand, they are quite vulnerable also to diagenetic alterations even after the death of the individual owing to the inner channels in its porous structure and an open morphological structure. This post-mortem assimilation of trace elements differs from element to element. Elements like copper, iron, lead and manganese are very likely to get incorporated in the bones due to the surrounding enriched soil of the burial ground (Carvalho et al., 2004).

Diagenesis has been discussed a lot in the present study as well as in various other literatures. The main obstacle in the interpretation of trace elemental data in archaeological remains, especially human remains, is that the elements of interest such as Fe, Cu, Mn, Zn, Sr, Ba and Pb and others are subjected to post mortem changes due to diagenetic processes during the burial period. They are related with the climate and geology of any site and can vary within the same site between different burials. Thus it is imperative to thoroughly assess each site independently of the other (Dudas et al., 2016).

The alteration due to diagenesis is not only in the form of change in the concentration of the trace elements but can also lead to the depleting or enriching of the levels of major elements such as phosphorus or calcium. This makes it vital to give due regard to the possible changes in the chemistry of human remains such as bones or teeth due to diagenesis before trying to make conclusions on the dietary reconstruction. Thus, the study of past food habits and bone chemistry requires a compulsory utilization of one or more of the several ways to assess the effects of diagenetic alteration in the remains. These methods include evaluation of Ca/P ratio, mutil-elemental correlational studies, evaluation of soil contaminants such as yttrium and zirconium, soil analysis to record the moisture and pH, comparison between different tissues in the human remains, and others. To be able to get more dependable results for the palaeodiet reconstructions, such methods provide a better understanding of the concentrations of different trace elements. The measurement of diagenetic changes is an advancing field with new approaches promising to bring in better assessment of diagenetic changes and the original biogenic signal in the human tissues (Price et al., 1992). Such researches have increased in the last few decades which try to record methods of monitoring and measuring diagenetic contamination in archaeological samples in order to neutralize their effects on teeth and bones (Nelson et al., 1986; Sillen, 1986; Price et al., 1992; Sillen & Sealy, 1995; Nielsen-Marsh & Hedges, 2000a, b; Hoppe et al., 2003). Sometimes the removal of diagenetic effect becomes inevitable but still the situation can be assessed and improved by using suitable cleaning and sampling procedures.

Bones are the tissues that keep remodelling and regenerating throughout the life while teeth enamel is very static in nature once it has formed early in the life (Boskey, 2007). As has already been mentioned, the enamel is more resistant to diagenesis than bone tissues (Reynard & Balter, 2014).

Contamination of bone in the ground embraces both physical and chemical forms. The airy shape of bone tissue is sensitive to infiltration by foreign materials. Calcium, for example, can be initiated through the precipitation of calcium carbonate in ground water. Hyphae (an algal growth), rootlets and fragments of charcoal are also perceived inside the bone structure as physical contaminants. Lambert et al. (1989) have advocated the use of mechanical abrasion to extract the outer surface of bone prior to examination as mentioned earlier. This preparation critically reduces the intensities of Zn, Cd, K. Al, Fe and Mn in bone but does not alter Na, Ca, Mg, Sr and Ba (Price et al., 1992).

Many researchers now suggest the use of dense cortical bone in contrast to more porous trabecular tissue to lower the effects of such physical contamination (e.g. Price et al., 1989). Trace element examination of archaeological bone has fundamentally disclosed three key groups into which elements fall:

a) Elements possibly introduced due to noteworthy diagenetic activity such as Fe, Al, Mn, Zn, Cu and Mg.

b) Elements which incline to leach out of bone with time such as Na and Mg.

c) Elements in bone primarily as biogenic signals such as Ca, Ba and Sr.

When possible, the test for a correlation between concentrations of different elements is a powerful tool for assessing the extent of diagenesis of a given element in a set of fossil samples. This is routinely done for C and N in bone collagen to assess the level of preservation of the bone and should be the case for Fe, Cu, Ba, Pb, Sr and other TEs (Jaouen et al., 2012; Pietruszka & Reznik, 2008) due to its simplicity.

All these above mentioned chemical elements and the correlations between them are being continuously explored in order to gain a deeper cognition of the dietary habits, modes of nutrition, palaeoenvironmental conditions and the changes occurring in them through space and time. Theoretically, this observation has become a rule to base research on elemental concentration in osseous remains and its relation with the socio-economic standing of the individual in ancient populations (Szostek et al., 2003).

3.7 Trace Element Detection and Quantification using ICP-MS

There are a number of techniques in the literature (Bolann et al., 2007; Zwanziger, 1989) which have been used in the quantitative and qualitative assessment of trace elements in human remains such as Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Yoo et al., 2002; Kniewald et al., 1994), Atomic Absorption Spectroscopy (AAS) (Martinez Garcia et al., 2005; Ericson et al., 1991), X-Ray Fluorescence (XRF) (Carvalho et al., 2004), but most of these are not able to identify and quantify multiple elements simultaneously using only a very small amount of samples in a large range of variable concentrations. This is when Inductively Coupled Plasma-

mass spectrometry (ICP-MS) comes in handy as it is capable of such kinds of analyses (Helliwell et al., 1996; Grattan et al., 2002).

ICP-MS is a widespread and routine analytical technique that has been used for determination of trace elements since the 1980s. It delivers quick quantitative analysis and allows to find out more than 70 elements with good accuracy. Other techniques such as AAS, ICP-OES, NAA have been replaced by ICP-MS since the first instance of its commercialization in 1983 because as has been already mentioned it offers a swift multielemental trace element analysis in a wide range of concentrations with very small amount of samples. Except elements such as hydrogen, oxygen, carbon, nitrogen, chlorine, fluorine, argon, helium, neon, and others, it can detect almost all other important elements with the detection limits sometimes even up to parts per trillion (ppt). The technique is quite fast with its output of the results of analysis with each sample not taking more than 4-5 minutes depending on the number of elements or their isotopes required to be analysed.

As a result, it is understandable that this sustained application of ICP-MS has given a boost to the examination of trace elements of biological origins (Tandon et al., 1998). In case of ICP-MS it is imperative to firstly optimize the instrument properly before starting the measurements since it makes sure that the measurements will be more precise and accurate (Yang, 2009). It has already been applied successfully for TE analysis in bones and teeth in many studies (Allmae et al., 2012; Szostek et al., 2009; Dolphin et al., 2013; Liu et al., 2013; Lohne & Agelarakis, 2014). Nonetheless there are some constraints to the use of ICP-MS due to the non-availability of matrix matched standard reference materials in case of tooth enamel as well as the long time period and elaborate procedures required for the preparation of samples. Additionally, ICP-MS only gives bulk composition of the analytes but spatial resolution and in depth layerwise analysis cannot be performed using ICP-MS but is possible if it is coupled to a laser ablation system (LA ICP-MS).

4
Objectives

In the present study, the characterization of the bones and teeth samples from the burials in the middle age monastery of San Pablo in Burgos has been explored to understand the living food habits and post mortem diagenesis in some of the remains using TE analysis. The macro and micro-elements present in the archaeological teeth and bones in this research have been studied using ICP-MS to find the possible contribution of these elements for getting insights into the historical samples.

Some of the individuals included in this study have previously been subjected to microwear analysis for diet reconstruction. Therefore, the microwear analysis results can provide an additional and complementary verification for the results obtained from TE analysis about the diet of these individuals. Hence, the microwear results have been included in this work to make comparisons with the results obtained.

The reliability of studies based on the examination of a single tissue type has always been questioned by researchers (Pankowska et al., 2016). The objective of the current investigation is to explore whether the concentration of trace elements in human bones and tooth enamel can reveal environmental conditions, dietary habits and assimilation of some elements from the surrounding soil. The intention of this study thus is twofold. First, inferences on food habits which aim at both the overall nutritional pattern and intra-populational dietary variations based on gender, status or chronology, which becomes clearer with the help of cluster analysis. Secondly, a comparison between the two different kinds of tissues in human bones along with tooth enamel with a view towards susceptibility to diagenetic changes.

It is aimed to explore a new methodology to be able to quantify or at least recognize diagenetic alterations in the elemental levels. Not only the quantification but first and foremost to realize which TE levels are the most susceptible to diagenetic modifications and to which extent. This might be done by comparing TE levels between different types of tissues i.e. cortical bones, trabecular bones and tooth enamel in this case, and identifying significant differences in the concentrations. The difference between the concentrations of TEs in bone (spongy and compact) and dental tissue (enamel) may follow three possible factors:

a) Element proportion in trabecular and cortical bone, and enamel may rebound the specific responses of each tissue to sampling.

b) Levels may vary due to the heterogeneity of each element inside of living tissue caused by an individual's development, specific way of metabolism, tissue mineral incorporation, disagreement in the elements' absorption or age-dependent changes (Dolphin et al., 2005) and diseases (Alvira et al., 2011; Gemmel et al., 2002; Malara et al., 2006).

c) Each tissue is temporary influenced by diverse post-mortem diagenetic alterations. Bone tissues break up more quickly than enamel, which is known to be less sensitive to diagenesis (Copeland et al., 2008). However, enamel is not entirely resistant to diagenetic action over longer time scales – similar to that of fossilised remains in paleo-anthropological contexts (Sponheimer & Lee-Thorp, 2006).

The objectives of the current study can thus be summarised as follows:

a) To get acquainted with the sample preparation procedures and elemental analysis regarding archaeological human remains and ICP-MS;

b) To make inferences on the diet of some individuals buried in San Pablo Convent using TE analysis and to compare them with previous literature as well as with microwear analysis;

c) To explore the establishment of a methodology for recognizing diagenetic uptake using different kinds of skeletal elements- teeth enamel, cortical and trabecular bones from the same samples;

d) To find possible relations in diagenetic and metabolic pathways between different TEs;

e) To explore the development of a cost-effective methodology for making in-depth research into palaeodiet and diagenesis using human remains from sites where archaeological context cannot be revisited.

In this thorough study we represent our pilot research concerning the differences in trace elemental responses of different kinds of skeletal tissues (bones and enamel) to various external factors most specifically diagenesis. Contrast was expected among dental tissue and bone tissues. If this is not the case, then the difference relate to factors that are not related to diet or geography. Discussions have been build up using literature review in order to bring out the reasons behind such differences.

5
Materials and methods

The bone and teeth samples belonging to five individuals from the burials found in the cloister courtyard (UE-250) and three individuals buried in the church nave (UE-350) excavated from San Pablo monastery were selected to be analysed in this work. All of these individuals were fully grown adults whose exact age is not known. All the specimens studied here belong to the archaeological collection of the Medieval Monastery of San Pablo, located in the city of Burgos (Spain) and housed in the LEH of the University of Burgos. In addition to this, detailed anthropological information about the samples is still unpublished at the time of writing and thus not available to be included in the study at hand. The details are given in the table (Table 2) and the pictures of the samples can be seen in appendix I.

Table 2: Details of the samples analysed in this study.

San Pablo cataloge number	Number for the present Study	Gender
02.25/1-250/7579	SP-7579	Female
02.25/1-250/7575	SP-7575	Female
02.25/1-250/7533b	SP-7533b	Female
02.25/1-250/7581	SP-7581	Male
02.25/1-350/7535	SP-7535	Male
02.25/1-350/7525	SP-7525	Female
02.25/1-350/7544	SP-7544	Male
02.25/1-250/7568	SP-7568	Male

5.1 Bone sampling

Classification of any specified type of bone as an idiosyncratic sample for the whole skeleton emerges to be far from being sufficient. For the interest of gaining an ample insight into the dispersion of various trace elements in different types of bones, it is essential to carry out controlled inspection on different types of bones (cortical and trabecular segments from the same sources) from the same skeleton under well determined sampling conditions (Tandon et al., 1998).

For minimizing impact to the skeleton, sampling intact bone should be avoided if fragmentary bone is available while selecting a bone for study. This was taken into consideration while taking the samples and already fractured and fragmented areas were used for collection of both spongy and compact bone tissues. Bone samples have to be extracted carefully because different bone tissues and their different parts may not be alike in their elemental composition (Smrčka, 2005; Pollard et al., 2007). Although the amount of enamel and bone needed for TE analysis is quite small (less than 1 g for traditional bulk sampling methods), there are ways of minimizing one's impact on archaeological materials when selecting prototype for sampling. It should be kept in mind that collecting samples for TE analysis contain the permanent subtraction of enamel and bone from archaeological specimens and samples which are entirely expended during digestion and ICP-MS.

5.2 Bone Sample preparation

Calcium and carbonate are among the most abundant ions in soil solutions which may desecrate bone as calcium carbonate or exchange ionically with existing hydroxyapatite to produce a carbonate apatite (Price et al., 1992).

The preliminary efforts to reduce or eliminate contaminants involved the expulsion of the outermost cortex and the inside surface of the bone by physical abrasion (Lambert et al., 1989). This abrasive cleaning significantly lessens the amounts of K, Fe, Al and Mn, which are abundant in soil oxides and clays but are markedly very low (< 200ppm) in fresh bone (Driessens & Verbeeck, 1990). Studies (e.g. Henderson et al., 1983; Williams & Marlow, 1987; Williams, 1988; Williams & Potts, 1988) of the amounts and pattern of TEs that are common in groundwater but are absconded in biological bone (e.g. Y, U, Th and rare earths) indicated that bones are not just infected superficially, however, but can be pervasively influenced by absorption and cation exchange. Abrasive cleaning extracts much of the most intensely affected portions of the bone, along with adhering soil minerals, but it does not fully extract contamination that might have more prevalently infiltrated the bone. In summary, mechanical attrition of the surface of the bone is a key step in the successful control of diagenesis, but may not be sufficient. Given the well-preserved state of bones and visibly non-existent concretion and physical minerals attached to the bone, the mechanical cleaning was restricted to only the removal of 2-3mm external layer of the bone.

Due to the nature of the present study which also aims at establishing diagenetic differences in the concentrations of TEs through the comparison of their contents in teeth enamel, spongy and compact bone tissues, bone specimen were not subjected to any rigorous chemical cleaning. The only pretreatment given to the bones before drilling out the powder was the mechanical abrasion of the exterior surfaces for already mentioned reasons. It has even been recommended to take the samples from cortical bone as this type of bone is less sensitive to diagenetic transformation than trabecular bone. The nature of the present study nonetheless demands the extraction of bone samples from both kinds of tissues for later comparative results on diagenesis.

For acquiring the specimens of spongy and compact bones, femurs were selected as the sample collection part of the skeleton for each individual. Femurs can serve as a reservoir for TE data of until ten years before the death of an individual and were uniformly available for all the samples along with suitable fractured and damaged surfaces. Such surfaces were utilized to extract the samples so as to minimize damage to the integrity of the archaeological remains as far as possible. The compact bone was collected from the femur shaft in all the cases. For spongy bone collection, the most convenient and damaged areas were selected (Table 3).

Collection of bones for TE analysis was accomplished using a Dremel Multipro drill, generally ensemble with an inverted cone tip to extract bone powder or a diamond disk saw to extract bone. Prior to extracting the sample, the sample area was cautiously worn out to extract surface contamination chunks. It has been advocated that minimum 50 mg to 1 g of bone should be collected as some preparation protocols for bone are quite stern and can result in a partial loss of sample (Hoppe et al., 2003). Both the trabecular and cortical bones were drilled with the same tools. The drill bits were cleaned with distilled water and dried after every sample extraction in order to minimize any contamination within the samples. The sample powder for bones was weighed after extraction on digital balance with 0.01 g precision (Table 3).

5.3 Tooth Sampling

Most initial dental element data were acquired by fusing various kinds of teeth or pooling samples. Nowadays we know there are many idiosyncrasies in elemental composition for various tooth types. Brown et al. have suggested the use of a single tooth type (Brown et al., 2002).

Table 3: Details of bone samples collected.

Sample number	Amount of cortical (compact) bone (g)	Amount of trabecular (spongy) bone (g)	Area of extraction for trabecular bone
SP-7579	1.83	1.80	From inside the fovea
SP-7575	2.51	1.66	From the inner surface of the shaft
SP-7533b	2.29	1.40	Inside surface of shaft and from inside of the upper head
SP-7581	1.48	1.59	Inner surface of shaft and epicondyle
SP-7535	2.26	1.81	From inside the epicondyle
SP-7525	1.90	1.62	From inside the epicondyle
SP-7544	2.52	1.51	From the upper end of the femur
SP-7568	2.93	1.55	From inner surface of damaged shaft and epicondyle

For archaeological sampling of tooth enamel in case of dental remains it has been suggested to try to examine teeth that are no longer embedded in the alveolar bone if possible, as this cuts down the chances of damage to the surrounding alveolus and bones of the skull. Additionally, it was attempted to avoid removing enamel or bone specimens from those teeth or parts of the skeleton that shows pathological lesions, cultural modifications, or other diagnostic markers as these amenities can possibly be used to reassemble aspects of health, diet, growth, and socio-cultural practices midst ancient populations (for e.g., Larsen, 1997; Katzenberg & Saunders, 2008).

For the current studies, mostly second premolars have been used wherever possible to try to homogenize the tooth type as far as possible. This decision of using second premolar was based on its availability in most of the archaeological samples for destructive analysis. For two of the individuals, 1st molar and 2nd Molar were used since the premolars were not available for analysis (Table 4).

Table 4: Details of tooth enamel samples collected.

Sample number	Type of tooth sampled	Weight of the tooth (g)	Amount of tooth enamel acquired (g)
SP-7579	M2 (with dental caries)	2.28	0.38
SP-7575	M1 (with dental caries)	1.87	0.31
SP-7533b	P2	0.84	0.19
SP-7581	P2	1.09	0.18
SP-7535	P1 (with dental caries)	0.58	0.24
SP-7525	P2	0.60	0.16
SP-7544	P2	0.74	0.08
SP-7568	P2	0.76	0.13

5.4 Tooth sample preparation

As discussed earlier, tooth enamel is largely impregnable to diagenetic degeneration while archaeological bone is highly sensitive to post-mortem modification. As a result, pre-treatment protocols for tooth enamel are comparatively forthright and less deep than that necessary for archaeological bone specimens. While various groundwork procedures exist for archaeological tooth enamel, the common accord of washing overnight with weak (1.0 N) acetic acid to extract most of the diagenetic carbonates in enamel (Sillen, 1986; Hoppe et al., 2003) was undertaken. Given the very small amount of enamel expected to be recovered, the teeth were washed before being powdered. It has been already warned that specimens can lose up to 70%age of their weight resting on the definite arrangement protocol followed (Hoppe et al., 2003). Thus, the whole teeth samples were subjected to acetic acid baths. The teeth was then cleaned by rinsing with distilled water. Specimens were left overnight to dry. Subsequently the teeth were weighed using a digital scale with a precision of 0.01g.

The enamel surface of the tooth for sampling was cut down using a Dremel tool and flat disc cutter to extract surface enamel. Dentin was manually separated from enamel whenever required. Enamel specimens were then broken down and consequently ground into a finer powder using a sterilized mortar and pestle. It was intended to collect approximately 5–20 mg of tooth enamel as has been

suggested in literature (Slovak & Paytan, 2012), although only a small amount of enamel is needed for actual examination. This is because some material may be lost during sample preparation. The enamel powder obtained was later weighed on the scale again (Table 4).

Finally, it should be of utmost importance to frame up hypotheses as multiple elemental and isotopic signatures can be recovered from a single dental or bony element and plan the sampling strategy so as to maximize the kinds of chemical facts that can be recovered from the samples while minimizing damage to the skeleton (Slovak & Paytan, 2012).

5.5 Sample preparation for ICP-MS

Approximately 0.5 g of bone sample powder for both compact and spongy bones and around half of the quantity of enamel powder obtained, which was usually not more than a few miligrams (Table 4), were digested in 8 mL concentrated trace metal grade Nitric acid (HNO_3 65%age Suprapur, Art. 1.00441, Merck KGaA, Germany) and 2 mL of Hydrochloric acid (HCl 30%age Suprapur, Art. No. 1.00318, Merck KGaA, Germany) in closed Teflon-TFM pressure vessels. These were left to digest according to a preinstalled digestion programme (Table 5) in an accelerated microwave digestion apparatus (ETHOS SEL, Microwave solvent Extraction Labstation, Milestone microwave laboratory system). The digested samples were then taken out after cooling down to the room temperature and diluted with 14mL of MilliQ water (H_2O). This base sample solution was stored in clean polypropylene vials. The weights were recorded independently at each step with a digital scale of precision of 1 mg. Duplicates were prepared for each of the samples to make more precise measurements. Preparation blanks were made for each set of eight samples throughout the complete preparation procedure with the same volumes of reagents. Blanks are useful to monitor possible contamination due to sample preparation procedures. A total of ten blanks were prepared for 48 samples.

5.6 Semiquantitative and quantitative analysis using ICP-MS

The semiquantitative mode in ICP-MS is used when the composition of the analytes is not known or only limited information is available. It was used for a preliminary screening of the composition of the samples in order to be helpful in the later quantitative analysis. The samples were diluted to approximately 3 times of the base solution using 2%age HNO_3 at first in order to perform a semi-

quantitative analysis for the desired materials. The total dissolved solids (TDS) in the solution for analysis by ICP-MS has to be kept less than 0.1%age and the final concentration of acid has to be less than 5%age in the solution. This semiquantitative analysis without the use of any Certified Reference Materials (CRM) is done to find out what elements are present in the samples and to what level of concentrations. Taking this as the basis, further dilutions were prepared for the quantitative analysis in order to detect the elements according to their concentrations in the sample so as to keep the concentration of the desired element in the samples less than 200 ppb to fit the range of the calibration curve. A general overview of the different elements and their concentration was gathered out of which ^{31}P, ^{44}Ca, ^{55}Mn, ^{54}Fe, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{138}Ba and ^{208}Pb were selected for further quantification. The digested samples were later processed through a series of dilutions from the base solution for analysis of different groups of elements with different concentrations in different samples. Accordingly, the results were normalized using the respective dilution factors.

Table 5: Digestion program used for the samples.

Temperature	**Time (minutes)**
T(amb)-80º C	4
80º C - 120º C	4
120º - 180º C	5
180º C	30
Ventilation	60

Calibration standards were prepared from serial dilutions of multi-elemental standard solution by Merck KGaA (1000 mg/L, 23 elements in 6.5%age HNO_3) traceable to various NIST Standard Reference Materials (SRMs) respective to each element with certified values for Ca, Fe, Mn, Cu, Zn, Sr, Ba and Pb. For phosphorus, Merck phosphorus ICP standard 1000 ppm solution was used with certified value (Table 6). The calibration standard solution concentrations ranged from 0 to 160 ppb (0, 2, 5, 10, 20, 40, 80, 160 ppb) to fall within the linear range for the instrument, using approximately 1%age ultrapure HNO_3. One calibration blank and at least five calibration standards were used to establish each analytical curve. Quality control standards (QC) were made

in the same acid matrix as the calibration standards at concentration near the midpoint of the linear range for each element analyzed.

Table 6: The NIST SRMs and Recovery percentages for all the elements analysed.

Element	NIST SRM No.	Recovery (%age)
P	SRM 3139 a	98.8±8.5
Ca	SRM 3109a	102.3±15.9
Ba	SRM 3104a	94.8±4.2
Sr	SRM 3153a	89.6±6.9
Zn	SRM 3168a	91.8±7.7
Cu	SRM 3114	93.3±6.5
Fe	SRM 3126a	90.5±4.1
Mn	SRM 3132	91.3±6.1
Pb	SRM 3128	93.4±1.8

The analysis was run at no gas mode available with the ICP-MS (Agilent 7500 ce, Octopole Reaction system) using an autosampler (CETAC Technologies, ASX-500, Model No. 510) until predetermined acceptance criteria (recovery percentage of 70-130%age, precision of RSD<10%age and linearity of $R^2 \geq 0.99$) were no longer met. Prior to analysis, internal standard (ISTD) using ^{9}Be, ^{89}Y, ^{159}Tb, ^{232}Th were added on-line at the time of analysis. A minimum of three replicate scans were recorded for calibration standards, all QC and samples. The average result of all the multiple scans were used. The calibration curve was fitted using linear regression. The final concentrations have been taken as the average from both the sample and the duplicate of the sample.

A tuning solution containing 1 ppb of Co, Li, Y, Ce and Ti was scanned prior to calibration and sample analyses to demonstrate instrument precision, stability and identification of ICP-MS. Rinse blank solution consisting of 1-2%age HNO3 v/v was flushed through the system after every sample and standard analysed. The detection limit for P, Ca, Sr, Fe, Mn, Zn, Pb, Ba and Cu was respectively 0.15, 0.16, 0.007, 0.056, 0.0027, 0.075, 0.0047, 0.003 and 0.007 ppb.

5.7 Comparison with previous literature

The obtained levels of the elements in question have been compared with previous studies including different methods of elemental analysis with samples belonging to different communities

and time periods in order to extract any possible inferences on the food habits. Ba/Sr, Zn/Ca, Ba/Ca and Sr/Ca ratios have also been compared with literature values.

5.8 Statistical analysis

A number of statistical tools have been utilized to process the data obtained from ICP-MS and make observations on the various aspects related to the diet of the individuals and the in-vivo or diagenetic uptake of elements in the samples. The statistical work was done using NCSS 11 Data Analysis software and Data Analysis Toolpak in Microsoft Excel 2013.

5.8.1 Regression analysis

Regression analysis was used to explore significant correlations between all the elements in all the three kinds of tissues. Significant correlations ($p \leq 0.05$, confidence level $\geq$ 95%age) have been reported and discussed in detail. The relationships have been explored in order to find out any correlation among different elements in case of metabolic/diagenetic pathways or other antagonistic/synergistic relationships. Regression analysis has also been used to correlate log Ba/Ca and log Sr/Ca values for all the three kinds of tissues not only to make inferences on diet but also to find the proficiency of skeletal tissue examination in the reconstruction of past diets. The differences between bones and dental tissue need to be understood to find out the best one for palaeodiet analysis (Pankowska et al., 2016). Distinction among dental and bone tissues is anticipated. The properties of each tissue type coupled with a lack of solid standard reference materials, can cause the different answer of TE signals and misunderstanding of the data. Thus, in order to find the most effective skeletal element for diet inferences based on Ba, Sr and Ca levels, regression analysis can be used.

5.8.2 Two-tailed t-test assuming unequal variances

In order to find statistically significant differences among TE levels between genders as well as between the two different stratigraphic units (from different chronologies and possibly different socio-economic status as well), two-tailed t-tests were performed with the null hypothesis being that the means are equal among each pair of gender and statigraphic units. The test was performed for each element in case of all the three types of tissues. The confidence level was taken at 95%age.

5.8.3 Scatter plots

Scatter plots have been produced to compare concentrations for each TE among all the three distinct skeletal tissues and possible reasons have been discussed. It helps to distinguish between variability in the elemental levels according to the parent tissue.

5.8.4 One way Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD)

Quantitative tests for diagenesis have already been suggested which include measurement of dense, long bones to relatively more airy ribs from the same individuals. In the same kind of bone tissue, there is little variation in composition among different bones of the same individual. Since diagenesis can partially affect porous and dense bones, distinct composition between cortical and trabecular bone tissues for an individual suggest post depositional alteration (Grupe, 1988; Lambert et al., 1985).

The novel approach of using trabecular and cortical tissues from the same bone in every individual is being tried for the first time in this study. Femur has been used as the source for both trabecular and cortical bone tissues along with tooth enamel. ANOVA was performed for all the TEs to find out any significant differences among their levels with the null hypothesis being that the means for all the three tissues in case of each element are the same at a confidence level of 95%age. ANOVA has then been taken a step further by using Tukey's HSD in order to try to quantify the significant differences with confidence levels taken at both 95%age and 99%age.

5.8.5 K-means cluster Analysis

The levels for TEs considered as good dietary indicators were used to cluster the individuals into possibly distinct dietary preferences using K-means cluster analysis. Sometimes classifying data into clusters may provide more information about the results.

5.9 Microwear analysis for diet reconstruction

Dental microwear analysis is based on the relationship between patterns of dental microwear and the kind of food consumed by the organism and has been found useful through many studies (Perez-Perez et al., 1994; García-Gonzalez et al., 2015, Perez-Perez et al., 2003). There are two

kinds of surfaces namely occlusal and buccal which can be studied for these patterns. While occlusal surfaces have pits and scratches due to abrasion and tooth-tooth wear and are useful to distinguish between hard and soft diets, buccal microwear is usually only affected by abrasion and is capable of providing dietary information regarding meat and vegetal composition. With occlusal surface it is difficult to assess meat consumption as well as long term food habits due to its faster turnover rate than buccal surface.

It depends on the objective of the research to use either both the surfaces or any one desired. For this study buccal microwear data has been looked into which was available for only three samples but can provide information about the dietary habits of the individuals over a relatively longer period of time. The number as well as the length of striations is studied for buccal microwear patterns which is affected by the abrasive particles in the food and factors such as chewing force. Meat eating habits can be identified by recognizing longer and frequent vertical scratches whereas vegetarian diet produces more and longer horizontal scratches (García-Gonzalez et al., 2015).

The indices of the relative frequency of the horizontal and vertical scratches are compared in order to sort the samples into four dietary groups: 1) agriculturalist group, which is categorized by an entirely vegetarian diet; 2) hunter-gatherers from tropical environments, displaying a diet with a more intake of vegetal components than meat; 3) carnivorous hunter-gatherer and pastoralists, whose diet is primarily centered on meat and 4) hunter-gatherers from arid environments, who have a mixed diet. The three indices calculated i.e. the number of vertical and horizontal scratches divided by the total number of striations (NV/NT and NH/NT) and the number of horizontal striations divided by the vertical ones (NH/NV) are then used to plot and classify the samples into diet groups. (Perez-Perez et al., 1994, 1999; Lalueza et al., 1996).

The microwear features can also be entered into discriminant functions (DF) which display a good capability to discriminate between the aforementioned four basic diet groups. The DF scores that combine a number of variables are used to classify the samples. Angular and linear measurements are taken for all the microwear features on the buccal surface using digitized micrographs and Adobe Photoshop software (García-Gonzalez et al., 2015). The final plots obtained have been inferred and compared with the data obtained from TE analysis in the current study.

6

Results and discussion

6.1 State of preservation and other observations

Calcium and phosphorus are generally measured in order to evaluate the quality of the samples in terms of the preservation. A total of four male and four female adults constituted the samples (Table 2). The average percentage of Ca in the samples of cortical bones came out to be around 30.3± 2.5 %age with a greater variability in case of women rather than in males (variance = $9.5*10^8$ for females and $1.44*10^8$ for males). Thus there are gender differences in the contents of Ca in the samples but these are not statistically significant. The calcium content ranged between 25.5 %age – 32.4%age. The least percentage of calcium was found in the female individual 7525 out of all the archaeological samples. In case of trabecular bones the average calcium percentage was calculated to be 25 ± 4%age. The calcium percentage for enamel was calculated to be around 31.3 ± 2.76%age. The mean of phosphorus percentage for enamel, trabecular and cortical tissues is respectively 15.8±0.86, 11±2.13 and 13.7±0.82 %age.

The higher variability in Ca content among women might be explained by a higher variability in the level of diagenesis (Price, 1989). Women's physiological state also defines the calcium content in the bones of that particular individual. It is already known that the level of calcium in the bones of a woman might decrease during events of breast feeding, pregnancy or at the end of the fertile period of the woman's life (Allmäe et al., 2012). Unfortunately, the exact age of the samples is not known.

In order to describe the preservation state of the samples, the calculation of Ca/P ratio is important as it can indicate the state of the mineral matrix in the bone and teeth and its integrity. The average of the Ca/P ratio is 2·21 ± 0.11 for compact bones and 2.28 ± 0.13 for trabecular bones which agrees with the data that has been given in the published literature, (2·12 = El-Kammar, Hancock & Allen, 1989; 2·15 = Gawlik et al., 1982; Sillen, 1989; 2·16 = Katzenberg, 1984; 2.21 = Schutkowski & Hermann, 1999). The bones can thus be accepted to be in a good state of preservation. The Ca/P value for enamel came out to be 1.98 ± 0.18. Teeth enamel crystals have been reported to have a Ca/P ratio from 2.08-2.15 by different authors (Nylen et al., 1963; Cuisinier

et al., 1992; Patel & Brown, 1975). Table 7 gives the summary of Ca and P measurements. The values for each sample can be found in table 23, table 24 and table 25 in Appendix II.

Table 7: Summary of Ca and P measurements for all the three kinds of tissues.

	Tooth enamel	**Cortical bone**	**Trabecular bone**
Ca%age	31.29±2.75	30.28±2.5	25.07±4
P%age	15.79±0.86	13.70±0.82	11.04±2.13
Ca/P	1.98±0.18	2·21±0.11	2.28 ± 0.13

6.2 Comparison with previous literature values

The results for all the elements in the tooth enamel and cortical and trabecular bones have been compared with data from already published literature. The techniques, chronology and sample preparation differ from one research to another and therefore there is a great variability in the elemental data published.

Table 8: Comparison of concentrations (ppm) found in current study with previous studies for tooth enamel.

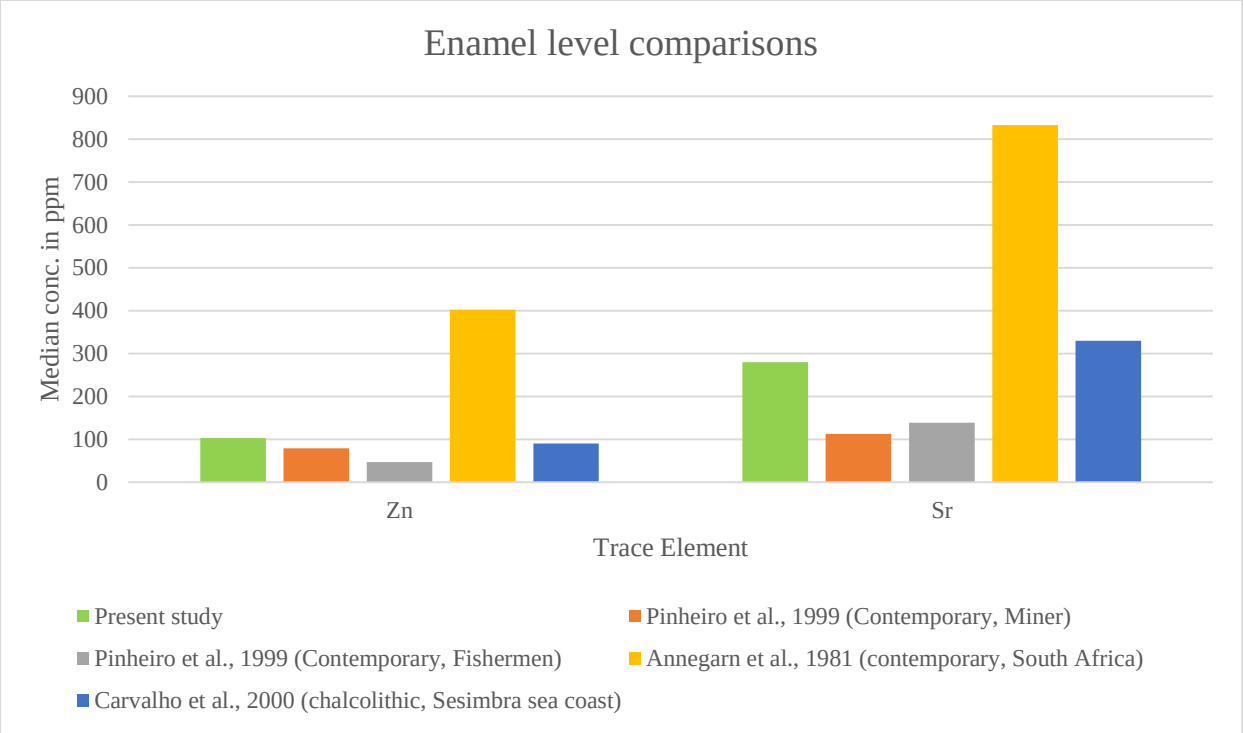
Element	**Mean from present study**	**Carvalho 2007 (Middle ages, South coast Portugal)**	**Carvalho et al., 2001 (contemporary, Azores islands)**	**Carvalho, 2004 (Neolithic, Portugal)**	**Liu et al., 2013 (contemporary, Taiwan)**	**Soares et al., 2008 (contemporary)**
Mn	5.9±4.8	30 ± 11	3.2 ± 2.1	37 ± 20		1.5±1.3
Fe	29.3±11.2	100 ± 80	11 ± 5	100 ± 40		
Cu	0.9±2.2	9 ± 5	2.1 ± 1.6	6 ± 2		
Zn	100.9±71.2	236 ± 60	150 ± 100	120 ± 50		202.6±124.1
Sr	363.9±177.3	350 ± 150	175 ± 35	120 ± 55	107.6±37.3	285.8±181.7
Ba	10.1±3.3	120 ± 70			1.9±1.0	
Pb	0.3±0.2	40 ± 20	2.1 ± 1.3	0.5 ± 0.2	0.8±0.5	

The two observations common for the teeth enamel (Table 8; Table 9, Appendix II) and the bones (Table 10) are the lower levels of Pb and Cu when compared with the values published in the literature from the contemporary experiments. Strontium levels in the tooth enamel have been found higher than those found in contemporary fishermen by Pinheiro et al. (1999) (Figure 7 a) and those found in the Middle Age remains from the South coast of Portugal by Carvalho (2007) (Table 8). However, they are lower, if compared with the Sr levels found by Carvalho (2000) in the chalcolithic remains from the sea coast site of Sesimbra, Portugal (Figure 7 a). For the rest of the elements namely Mn, Fe, Zn, Ba there is a great variability within the expected ranges of concentration from the information found in the literature.

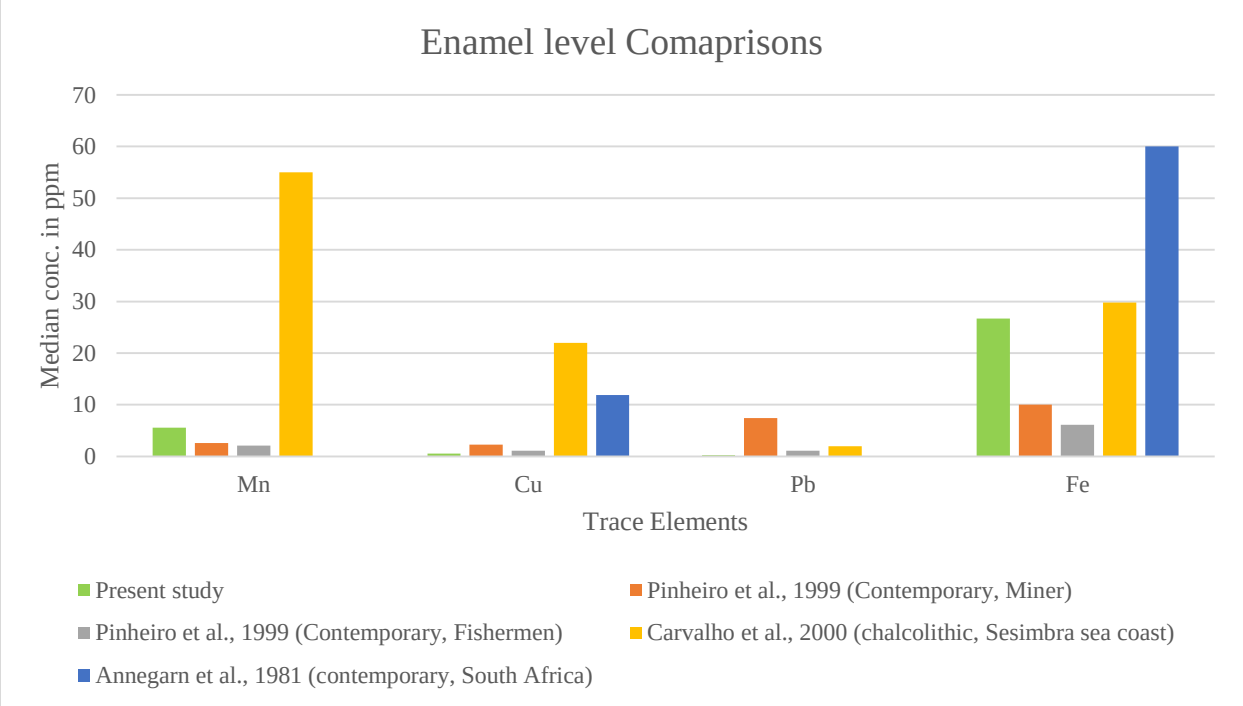
In case of compact bone, the Sr concentration was found to be lower than those found by Carvalho (2000) from the sea coast of Sesimbra. The rest of the elemental levels were also comparable except low levels of lead (Table 10). Zinc levels were found to be comparable to the literature values except they were much lower than those found by Annegarn et al. (1981) in their study in contemporary South Africa (Figure 7 a).

Table 10: Comparison of literature values of various elements with the current study for compact bones (ppm).

Element	Median	Range	Carvalho et al., 2000 (chalcolithic, Sesimbra sea coast) (Median)	Janes et al. (cited in Carvalho et al., 2000) (Median)	Carvalho et al., 1998 (contemporary) (Mean)
Mn	4.46	1-14	37	4.6	≤4
Fe	32.61	14.3-441.6	227	95.6	153±265
Cu	8.88	2.7-17	4.7	6.6	4.9±0.6
Zn	116.73	96-195	82	147.1	172±26
Sr	1220.49	757-2091	1352		147±55
Ba	85.38	33-553			
Pb	0.47	0.14-0.90	8.8		25±17



a



b

Figure 7 (a, b): Comparison of literature values of various elements with the current study for tooth enamel (ppm)

In case of the concentration of lead (Pb) in the bones, there is not a great variability in the results (Table 23; Table 25 Appendix II; Table 26 Appendix II). Usually, in the towns and cities of Middle Ages and early modern times, Pb is found in the samples due to the use of lead pipelines for the transport of water and also the use of kitchen utensils covered with lead glaze (Smrčka, 2005). The reason might be that the population under study in this research was not urban in nature and therefore were most probably not using lead pipelines or lead glazed cooking vessels. This low level Pb as compared to the previous literature (Figure 7 b) might altogether be the result of post-mortem uptake which cannot be excluded from the consideration since Pb ions have been found to pollute archaeological materials (Zapata et al., 2006). Even after such considerations, the value of Pb is quite low which indicates that the living conditions in that period were relatively free from Pb. Even in case of teeth, lead varies between 0.10 - 0.43 ppm.

6.3 Regression analysis

Statistically significant correlations between different TEs are reported in detail in this section. Based on the kind of tissue displaying the correlation, the metabolic or diagenetic relationships can be speculated for different pairs of elements. Apart from these, two types of relationships occur among the TEs, namely antagonistic and synergistic. Antagonism exists at two levels, absorptive and metabolic. At the absorptive level it occurs when the excess of one element can inhibit the absorption of another element in the intestines (Watts, 1990). For example excess intake of Zn is known to reduce Cu absorption (Davies, 2013). At the metabolic level, antagonism occurs when the excess of one element starts to interfere with the metabolic functions of another element or simply displaces it. Examples are Zn and Cu, Mg and P (Davies, 2013). Synergism exists among elements usually on a metabolic level. For example, Fe and Cu are synergistic because Cu is required in sufficient amounts for the utilization of Fe (Prasad, 2013). Many such antagonistic and synergistic relationships are present in the human body.

6.3.1 Correlations between elemental concentrations

Calcium displays a very strong correlation with phosphorus for spongy bones which indicates a strongly synergistic relationship (Figure 8 a). The synergistic relationship between calcium and phosphorus has already been mentioned by previous researchers (Watts, 1990). It also displays a

significant positive correlation with phosphorus levels in compact bones as expected (Figure 8 b). In case of tooth enamel, however, no significant correlation was found (Figure 9).

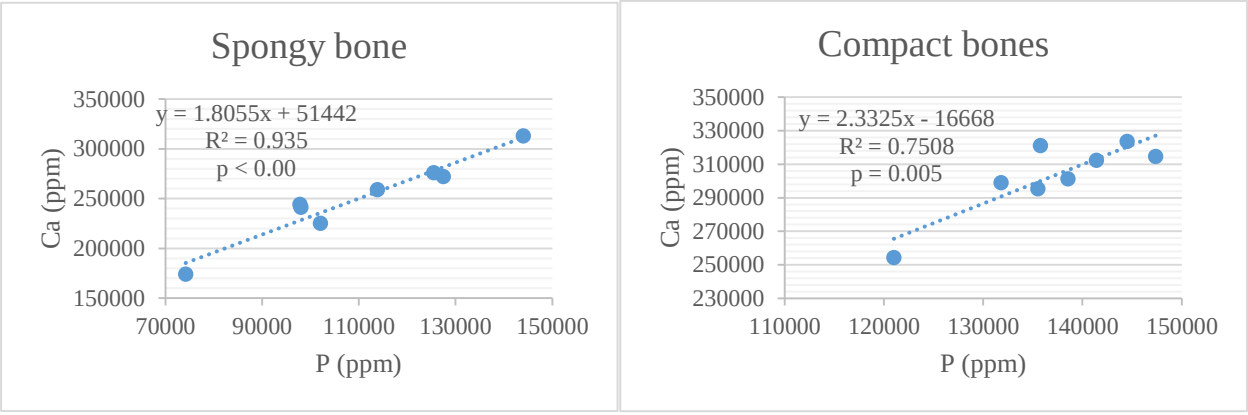


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Figure 8 (a, b): Correlation between Ca and P concentrations (ppm) in spongy bones and compact bones

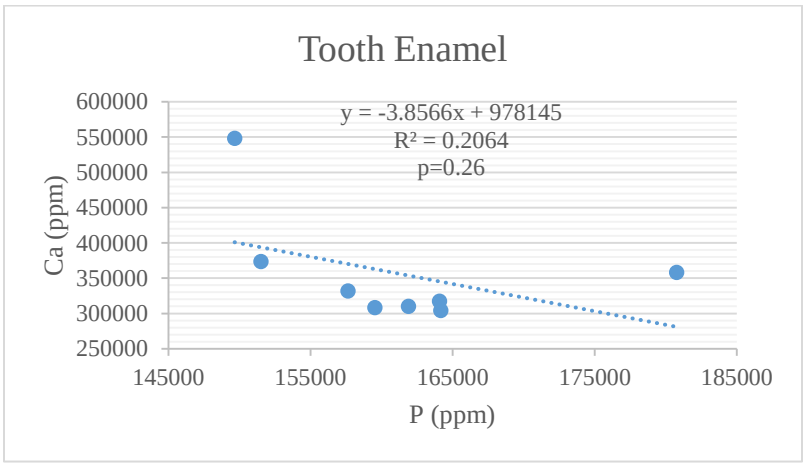

Figure 9: No correlation between Ca and P concentrations (ppm) in tooth enamel

Ca also showed inverse correlation with Cu, Ba and Fe in spongy bones (Figure 10 a, b; Figure 11 a). P also displays similar relationships with the above mentioned elements since it is strongly correlated with Ca in the trabecular bones (Figure 11 b; Figure 12 a, b). Additionally, P also shows inverse relation with Mn which was not statistically significant in case of Ca and Mn (Figure 13). These correlations can be explained as antagonistic but it is interesting to note that these are only displayed in trabecular bones and not in cortical bones or tooth enamel.

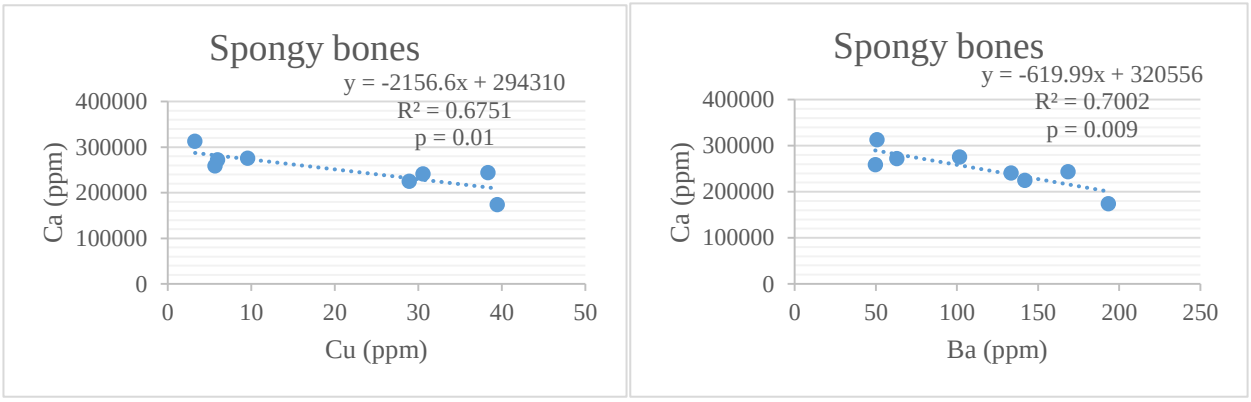

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Figure 10 (a, b): Inverse correlation of Ca with Cu and Ba in trabecular bones

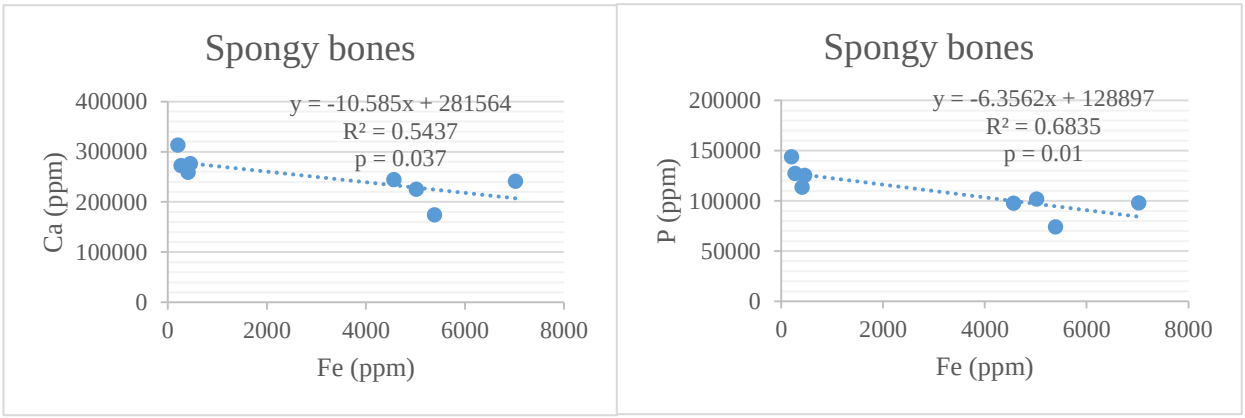

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Figure 11 (a, b): Inverse correlation of Ca and P with Fe in trabecular bones

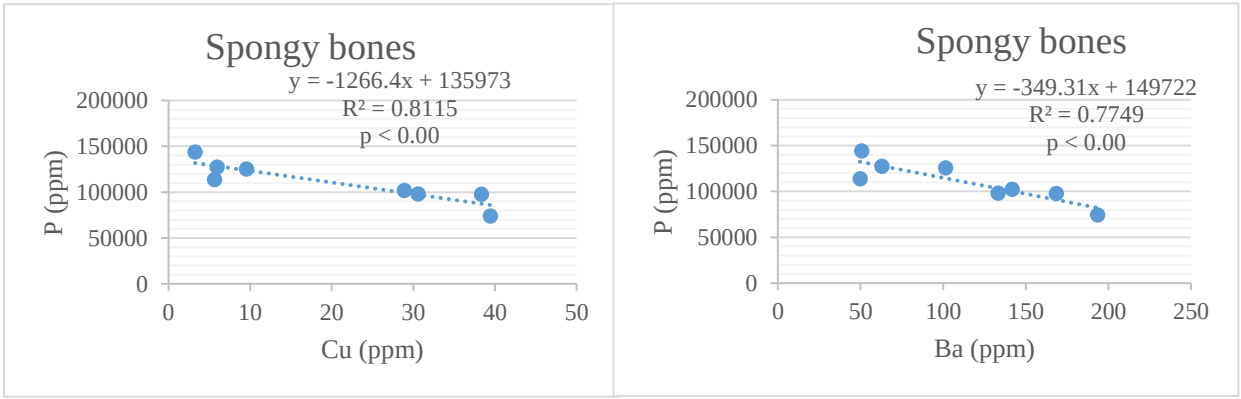

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Figure 12 (a, b): Inverse correlation of P with Cu and Ba in trabecular bones

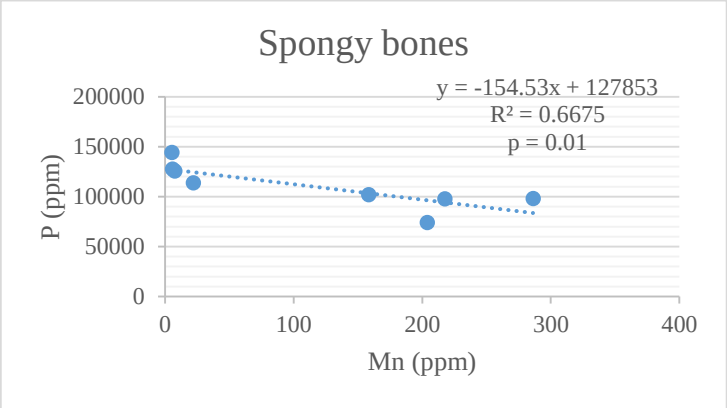

Figure 13: Inverse correlation of phosphorus levels with manganese

Iron levels in case of spongy bones and compact bones had a positive correlation with the Mn content (Figure 14 a, b). The regression coefficient is quite strong proposing an almost linear relation between Fe and Mn in spongy bones. Therefore strong association of Fe and Mn in this case as a diagenetic marker is suggested. The correlation can be explained due to the diagenetic uptake of Mn through the soil during burial due to association of Mn with Fe to form Fe-Mn oxy-hydroxides. This is not the first instance where Mn ions have been found to contaminate bones (Zapata et al., 2006).

This relation vanishes in case of teeth enamel as was expected. The regression coefficient is not strong enough to show any linear correlation between Fe and Mn for the tooth enamel (Figure 15). Thus in case of bones, the levels of Mn seem to be associated with the chemical processes of diagenesis which occurred post mortem. Therefore, the Mn content in enamel appears more reliable. The Mn concentration in the enamel varies from 1.7 to approx. 17 ppm.

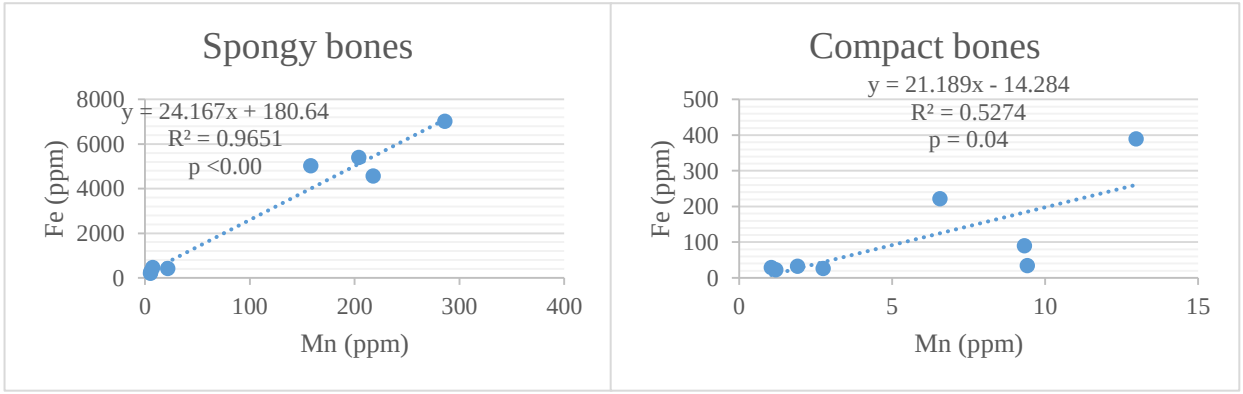

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Figure 14 (a, b): Correlation of iron with manganese in trabecular and compact bones

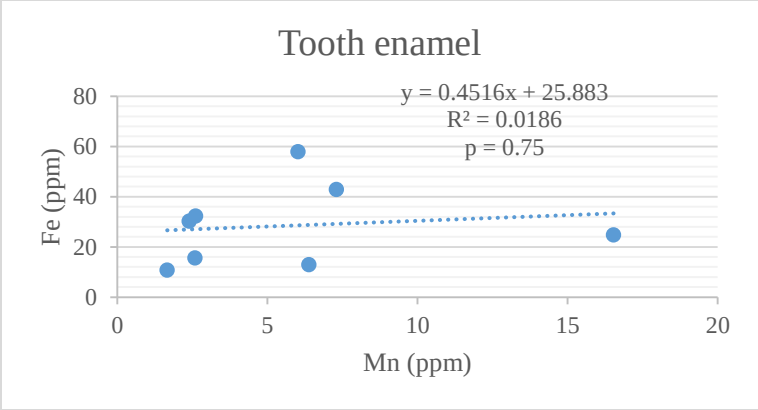
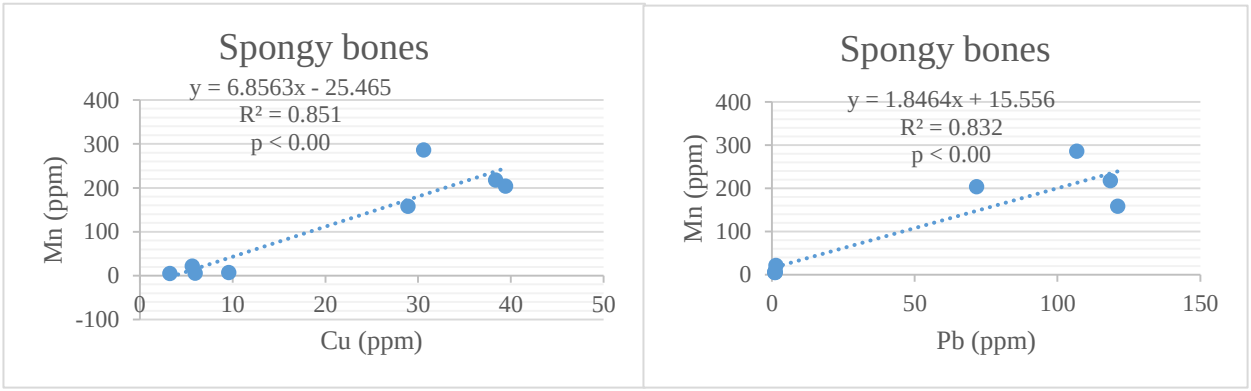


Figure 15: Iron does not display any correlation with manganese in tooth enamel

The levels of Mn have strong correlation with Cu, Ba and Pb in spongy bones which might point towards diagenetic uptake through a similar pathway for these elements (Figure 16 a, b; Figure 17 a) or to synergistic relationships. It has already been established that Mn in the trabecular bones is greatly affected by diagenetic alteration. Quite possibly complex compounds of these elements might exist which are taken up by the bone during burial. Ba also shows a very strong correlation with Cu in trabecular bones, further strengthening the case of Ba uptake from the soil post mortem since Cu in trabecular bones is speculated to be associated with diagenesis (Figure 17 b).



a b

Figure 16 (a, b): Mn correlation with Cu and Pb in trabecular bones

This might point towards Cu and Ba following similar pathways into the spongy bones as far as diagenetic uptake is concerned. This may even be in the form of compounds with divalent ions of both these elements. It can also point towards strongly synergistic relationship between Ba and Cu and Mn. The cortical bones also show a synergistic relation in case of Ba and Cu (Figure 18 a).

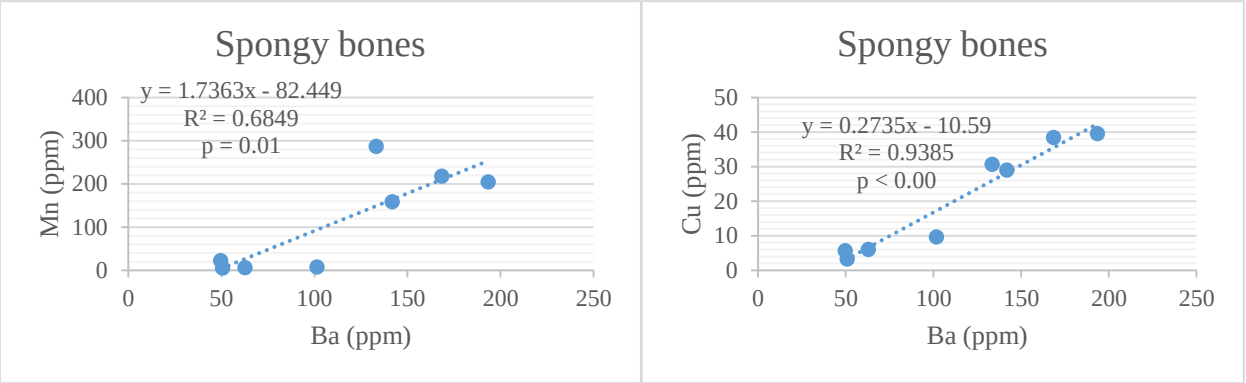


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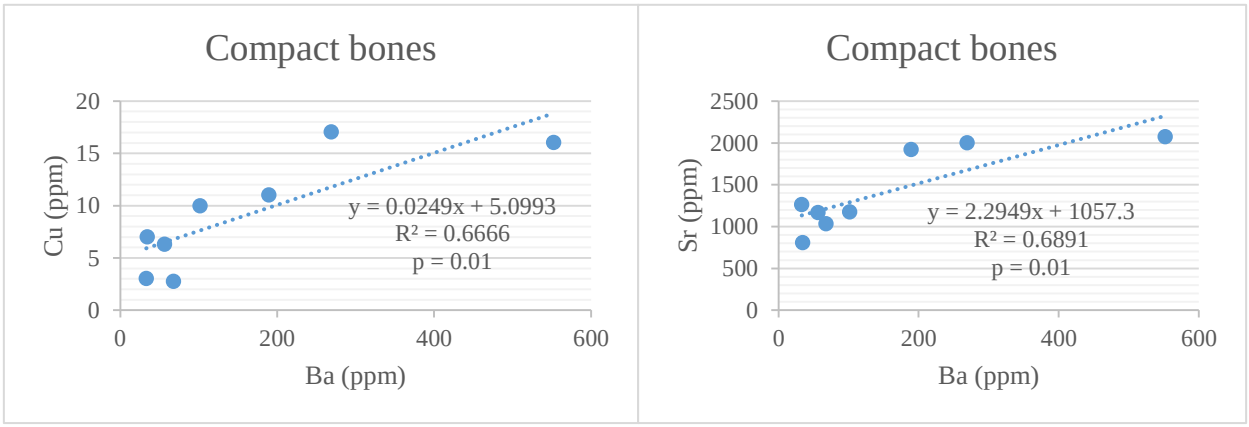
Figure 17 (a, b): Ba correlation with Cu and Mn in trabecular bones

In case of cortical bones, barium shows a significant correlation with strontium (Figure 18 b). This might point towards metabolic or synergistic relationships between these elements.



a b

Figure 18 (a, b): Ba shows a positive relation with Cu and Sr in the compact bone tissues

The Cu level in the spongy bones averages to around 20ppm with some samples having really high ppms of Cu which might be due to diagenetic uptake of Cu during burial in the highly porous spongy bones of the individuals. Cu displays strong correlation with Fe in case of spongy bone tissues (Figure 19 a).

Cu-Fe complex compounds are generally found naturally in geological settings and could easily be a part of the uptake mechanism of trace elements by bones from surrounding soil. Iron is known to form colloidal phases that can entrain massive quantities of copper and zinc and can be transported by groundwater (Jaouen et al., 2012).

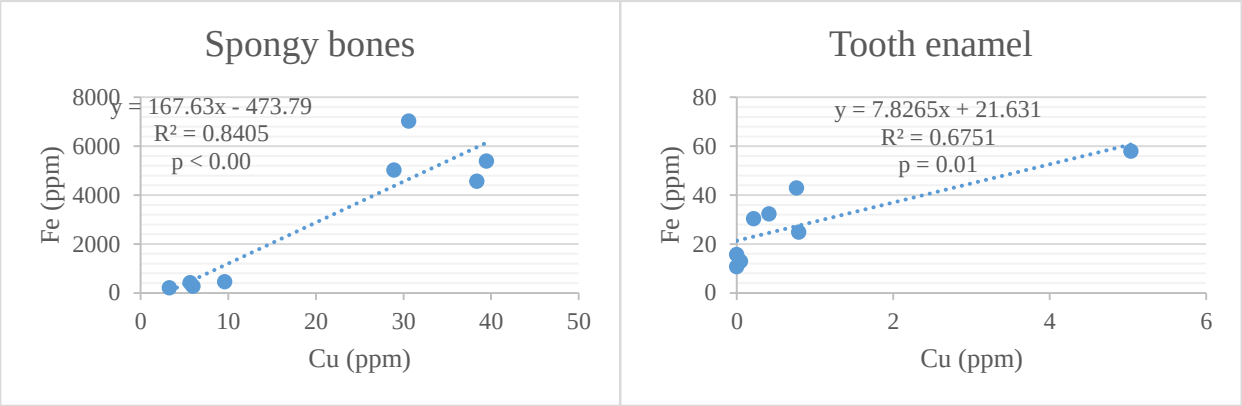


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Figure 19 (a, b): Cu correlation with Fe in trabecular bones and tooth enamel

In case of tooth enamel, Cu shows a positive correlation with Fe (Figure 19 b). Brian Kirkham (2013) in his study found the strongest relationship between iron and copper in human teeth which is an indicator of how Fe and Cu in their divalent form have similar progression making their way in the enamel of tooth. Their chemical similarity makes sure that they share many of the metabolic reactions in the human body (Kirkham, 2013).

Lead displays a strongly positive relation with Mn (Figure 16 b) as well as Fe (Figure 20 a). It might steer to the conclusion that lead follows similar pathways during diagenetic uptake as Mn and Fe and the three are closely associated with each other in this process. Zinc also displayed significant correlation with strontium in trabecular bones which might be synergistic in nature (Figure 20 b).

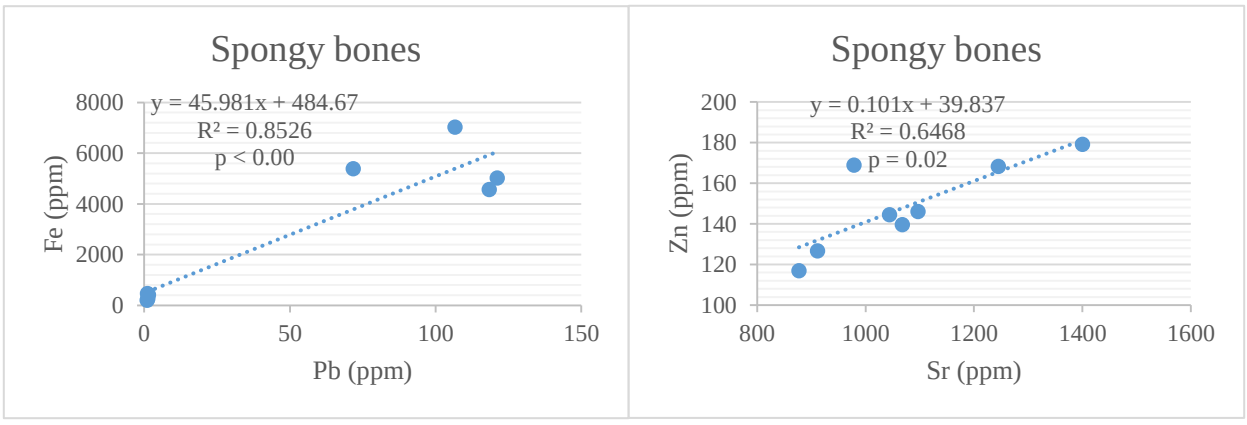

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Figure 20 (a, b): Pb correlation with Fe and Zn correlation with Sr in trabecular bones

6.3.2 Correlation between Ba/Ca and Sr/Ca ratios

Barium and strontium are usually utilized in comparison with calcium in order to make inferences on palaeodiet. In this case, the three different tissue types provide a large variability in the levels of Ba and Sr (Table 26, Appendix II) which makes it imperative to find out the most representative of these tissues when making inferences. More sensible interpretations hence include the comparison between different kinds of tissues to understand their effectiveness in palaeodiet reconstruction which is also attempted in this study by analyzing both bone tissues and tooth enamel. The correlation between Ba/Ca and Sr/Ca ratios for different tissues has been already attempted by researchers (Pankowska et al., 2016). With the growth in the understanding of diagenesis, tooth enamel are increasingly becoming the preferred material for the studies into palaeoenvironment and ancient dietary habits in which case it becomes imperative to perform experimental analysis work to relate Sr/Ca and Ba/Ca ratios in enamel and bones (Peek & Clementz, 2012; Austin et al., 2013).

This study takes it further by including trabecular bone tissues in the correlation. The ratios need to be correlated since they both indicate similar processes in each individual (Pankowska et al., 2016). The correlation was examined using regression analysis and was found to be significant in case of compact (Figure 21) and spongy bones (Figure 22) but in case of teeth enamel the correlation was not statistically significant (Figure 23). The correlation becomes weaker from compact to spongy bones.

The enamel diverges greatly from the spongy and compact bones ratios for both Ba/Ca and Sr/Ca ratio. The lowest points in the case of teeth enamel are the teeth from individual 7579 and 7575 (Figure 23) in whose case the 2nd molar and 1st molar respectively, were sampled while the rest of the individuals have been sampled with their premolars (Table 4). Therefore these low values of both the ratios in both these individuals in expected. More so for the first molar which has the lowest values of all since their mineralization starts from the birth approx. three years before the mineralization of the second molars (Table 1). The permanent second premolars start forming two years after the birth of the individual. Therefore the first molar could be influenced by lactating which brings it to a higher trophic level than the rest (Austin et al., 2013).

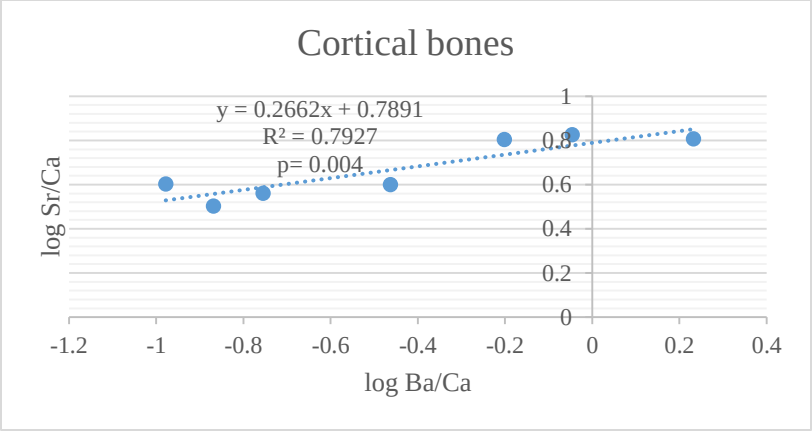

Figure 21: Correlation between log Sr/Ca (ppm/mg/g) and log Ba/Ca (ppm/mg/g) for cortical bones

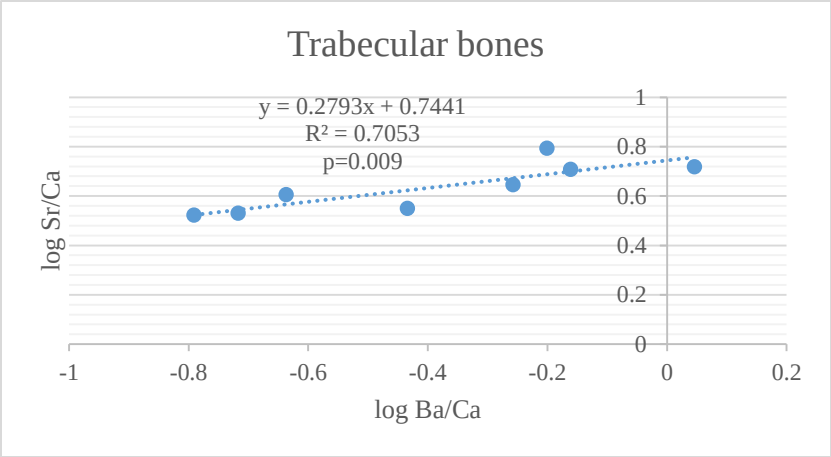

Figure 22: Correlation between log Sr/Ca (ppm/mg/g) and log Ba/Ca (ppm/mg/g) for trabecular bones

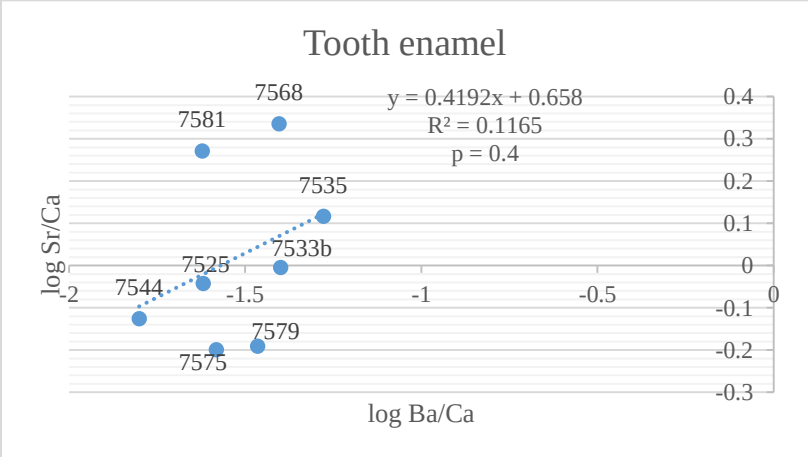

Figure 23: Correlation between log Sr/Ca (ppm/mg/g) and log Ba/Ca (ppm/mg/g) for tooth enamel

As has already been mentioned, the intake of TEs in the bioapatite mineral and its incorporation by the tissue varies from person to person and from tissue to tissue. While the enamel is in the process of mineralization, the absorption of Ca ascends compromising that of Ba and Sr which have much lower values in teeth than in bones (Balter, 2004). Therefore TE intake is much lower in the teeth enamel even when the organism is alive (Eggins et al., 2003).

Given the lack of correlation in case of tooth enamel due to non-homogenous nature of the tooth types or other factors such as individual absorption, time of calcification, individual metabolism and others, the Ba and Sr levels in tooth enamel were not preferred for making inferences on diet. Levels from cortical bone tissues have been used for all the TEs to discuss food habits except for iron and manganese, which have shown significant diagenetic uptake even in the cortical bones as already mentioned.

In summary, the regression analysis helped to select cortical bones for making inferences about diet. Tooth enamel was found to have more reliable results for Fe and Mn but not so for Sr and Ba. Elemental levels from all the three kinds of tissues were used to compare results to explore diagenetic uptake from the soil.

6.4 T-test for gender differences and differences between the burial areas

T-tests were performed for each element for both the tooth enamel as well as cortical and trabecular bones at a confidence level of 95%age to find possible gender differences or differences between the individuals buried in the two different areas of the church nave and the cloister courtyard.

6.4.1 Gender differences

Strontium was the only element that showed statistically significant differences in its concentration in tooth enamel among males and females (Table 11, Appendix III). The reasons for significant difference between the genders for Sr concentration in tooth enamel might be related to the individuals' metabolism or non-biogenic reasons. Li et al. (2013) has also found Sr variations based on gender in his work where females had more strontium levels than men which is not the case here. The Sr levels however also vary according to age as well as dental caries. Additionally, while Li has worked on contemporary Chinese men and women's teeth, in the current study of archaeological samples, diagenesis complicates the situation. The non-homogeneity of the tooth

type is another factor that affects Sr level differences. The amount of Sr was more in women's bones than men but the difference is not significant in terms of statistics.

In case of cortical bone tissues, no significant gender differences were found. Ba content vary greatly from 33 to 552 ppm among the cortical bone samples. The difference among the Ba average for women is approximately twice of that of men (Table 12).The difference among the two genders points to women consuming more plant food than men in their diet but are not statistically significant.

The gender difference in the levels of Mn for men and women is not statistically significant but women have greater amount of Mn and variability in the Mn content in their spongy bones (variance females = $1.8*10^4$, males = $1.3*10^4$) tooth enamel (variance females = 43.9, males = 7.28) both.

There are more than a single hypothesis to explain this higher level of Mn in the compact bone tissues of women. The direct inference is that perhaps women were consuming more plants than men but on a deeper level of understanding this might also be caused due to the difference in the physiology of women. This second hypothesis explains that women more often than men suffer from the lack of iron in their body due to their physiological specificity. Every organism that lacks iron tends to store greater amounts of Mn in its body (Finley et al., 1994). One or both of these hypotheses might be contributing to the higher Mn in the body of women. Consequently, iron is found to be higher in men than in women in case of both enamel and cortical tissues but not statistically significant. Here it's also interesting to note that the female individual 7579 with the highest Mn content of 16.5ppm is also very low on the Cu and Zn contents and thus maybe pointing towards more plant food and less meat and fish in her diet or other synergistic relations.

Cu and Zn levels indicate the fraction of meat and fish products in the diet of the people (Reynard & Balter, 2014). Cu does not display any significant differences among the women and men statistically. The range of Cu content in compact bones in both the genders is more or less similar and equal to the total average of about 9 ppm. Cu levels thus seem to produce complications in data interpretation since the levels do not show a great variability (Table 25, Appendix II).

On the other hand, the content of Zn in the women's compact bones is more than that in males. In the women's bones, the level of Zn is higher but the difference is statistically non-significant. The content of Zn ranges from 103 to 169 ppm in case of females in contrast to 100-137 ppm for males.

Alternatively, the female individual 7525 with the highest Zn levels in her tissues also has the lowest concentration of Sr which could indicate that she was consuming relatively less plant food and more meat than the rest of them. Therefore it could be a result of personal choice or her individual metabolism for these elements.

This can even be argued based on the fact that the Zn content in the bones of women increases with age while in the case of men's bones it decreases with time (Benfer, 1995; Magee et al., 1994). There is a large amount of variability in the Zn contents much more so in the case of females (Variance females = 1153.7, males = 314.2). This variability might be more understandable by further analysing the social status of these individuals. It may account to the different areas of origin of the community and its heterogeneity and how these differences are related with the different modes of nutrition.

Therefore a lot of care has to be exercised when interpreting the levels of Zn in bones. Additionally, Zn content in the bones is largely related to the organic fraction of the bone rather than to the inorganic fraction as a result of which the loss of organic matter in the bones can lead to more difficulty in the interpretation of Zn (Pearsall, 1989).

The gender differences do not display statistical differences among the levels of lead (Pb) in cortical bones which falls in the range between 0.14 to 0.90 ppms and is approximately at an average of 0.5 ppm for both men and women. This indicates that there was not a high level of Pb pollution in the San Pablo area.

Table 12: Table showing mean (ppm) for each trace element for Females (F) and males (M) in tooth enamel and cortical bone.

Element	**Mn**		**Fe**		**Zn**		**Cu**		**Sr**		**Ba**		**Pb**	
Tissue type	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Enamel	7.0	4.4	48.0	61.1	99.5	112.4	0.5	1.5	233.0	484.3	9.3	10.4	0.2	0.3
Compact bone	6.6	4.7	93.1	117.6	137.1	119.1	9.3	9.0	1516.7	1346.0	202.2	123.7	0.5	0.5

6.4.2 Differences between church and cloister samples

Tooth enamel has displayed significant difference in lead levels between the burials from the church and those from the cloister (Table 13, Appendix III). The samples from the church have significantly higher lead than those from the cloister. This could be related to the contamination from the burial place or a difference in lead uptake through the different time periods where the samples from the church are from a later time period than those in the cloister. The samples buried in the cloister courtyard have been conjectured by archaeologists to be of poorer sections of the society which might also indicate that in their rural living conditions they might not have access to lead glazed cooking vessels and lead pipelines which could be the case for higher lead levels in the samples from the church nave which most probably belonged to a higher class and might have lived in a more urban setting.

Significant differences were found in the levels of Strontium and Sr/Zn ratio for the cloister and church burials in cortical bones. The samples from the cloister courtyard display a higher Sr and Sr/Zn ratio value that indicates a diet with more plant food and lesser meat as compared to those buried in the church. The samples buried in the church have been conjectured to be from higher strata of the society as compared with those buried in the cloister given the fact that money had to be paid to be buried inside the church. However, they also belong from different chronological periods which could also mean a change in diet through the centuries. Therefore, in order to confirm diet differences based on social stratification, samples need to be analysed from both the burial areas belonging to the same time period. Inversely, burials belonging to different time periods from the same burial enclosure could also be compared to understand changes in diet structure through time.

Lead, copper, manganese and iron in trabecular bones have also shown significantly higher levels in case of samples from the cloister courtyard as opposed to samples from the church nave. This could be due to different burial/soil conditions in both the areas which possibly has contributed towards post mortem contamination. Other possible reasons for this difference have been discussed in later sections. The results are summarised in Table 14.

Table 14: Results for t-test between cloister courtyard and church nave samples from tooth enamel, cortical and trabecular bones.

Tooth Enamel	**Pb (ppm)**	**Ba (ppm)**	**Sr (ppm)**	**Zn (ppm)**	**Cu (ppm)**	**Mn (ppm)**	**Fe (ppm)**
Church (mean)	0.39	9.50	307.42	179.60	0.29	4.10	29.63
Cloister (mean)	0.19	10.06	389.36	70.80	1.22	6.64	27.74
T-test Significance	**Yes**	No	No	No	No	No	No
Trabecular bones	Pb (ppm)	Ba (ppm)	Sr (ppm)	Zn (ppm)	Cu (ppm)	Mn (ppm)	Fe (ppm)
Church (mean)	1.30	71.33	983.91	143.89	7.07	11.60	378.68
Cloister (mean)	83.85	137.50	1133.41	151.59	28.12	174.18	4439.53
T-test Significance	**Yes**	No	No	No	**Yes**	**Yes**	**Yes**
Compact bones	Pb (ppm)	Ba (ppm)	Sr (ppm)	Zn (ppm)	Cu (ppm)	Mn (ppm)	Fe (ppm)
Church (mean)	0.72	52.88	1004.38	142.22	5.36	3.22	91.97
Cloister (mean)	0.39	229.05	1687.57	119.64	11.42	7.10	113.40
T-test Significance	No	No	**Yes**	No	No	No	No

6.5 Scatter plot

The level of Pb increases from teeth enamel towards the highest in spongy bones. While in teeth the average concentration is 0.27 ppm, in the compact bones it increases to 0.51ppm and yet the variability in the samples is not very large. However in case of spongy bones, this variability increases with four samples displaying much higher level than the rest of the samples (Figure 24).

This can be explained by the enrichment of lead into the spongy tissues which have been lying in the direct contact with the soil leading to this diagenetic uptake of Pb. Further probing had to be made into the area of collection of samples for the four mentioned individuals which might explain the much higher Pb content. The samples were collected from the spongy bone at the inner surface of the shaft of the femur which was in direct contact with the soil while in rest of the individuals, the sample was collected from the spongy bone inside the upper or lower end of the femur bone (Table 3).

These higher concentrations could also be a result of the difference in the burial conditions or the soil composition in the two distinct areas as discussed earlier, but the lower levels in sample 7579 which also belongs to the cloister courtyard cannot be explained with this reasoning. Therefore, the area of extraction of the trabecular bone tissues seems to be the dominating factor for the exceedingly high level of lead in these four samples.

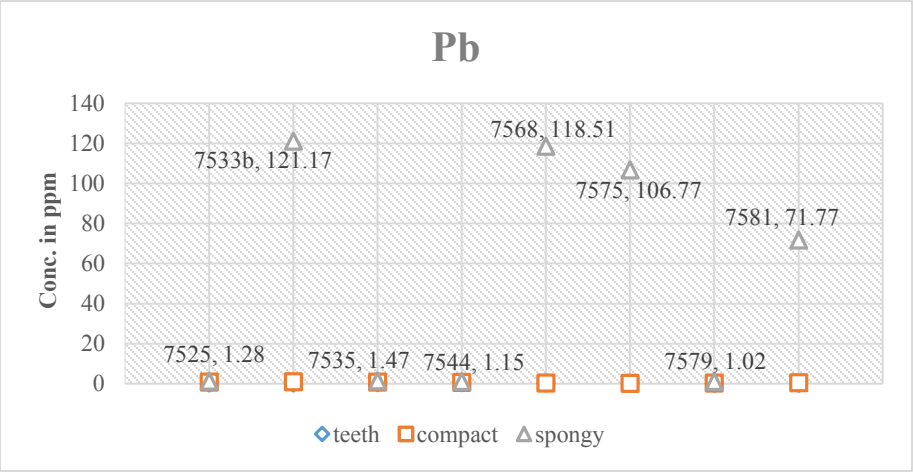

Figure 24: Pb levels in different tissues in different samples

The compact bones have not been affected much with the contamination due to Pb. In this case most probably the spongy bone was the first tissue to come in contact with the soil and the incorporation of the element starts from the outside towards the inside. It is well known that due to its harder structure, the compact bone is less vulnerable to diagenetic changes which occur post mortem to the remains and is therefore not penetrated by external elements to a very great extent. On the contrary, spongy bones are more susceptible to post mortem alternation due to a more open and porous morphology and the lead contents evidence to this fact.

No matter however much the use of lead pipelines and lead glazed cooking vessels was in vogue in the period of these burials, it does not explain such high levels of lead in the bones without including the diagenetic contamination through the soil (Rebocho et al., 2006). Therefore it is safe to say that the Pb levels in trabecular bones are of post-mortem origin.

The very same four spongy bone samples namely 7533b, 7568, 7575 and 7581 with highest Pb levels also are the ones with the highest Mn, Cu and Fe concentrations which reaches up to

1.2%age in case of the sample with highest Fe level (Figure 25, Figure 26, Figure 27). This level falls steeply in case of cortical bones.

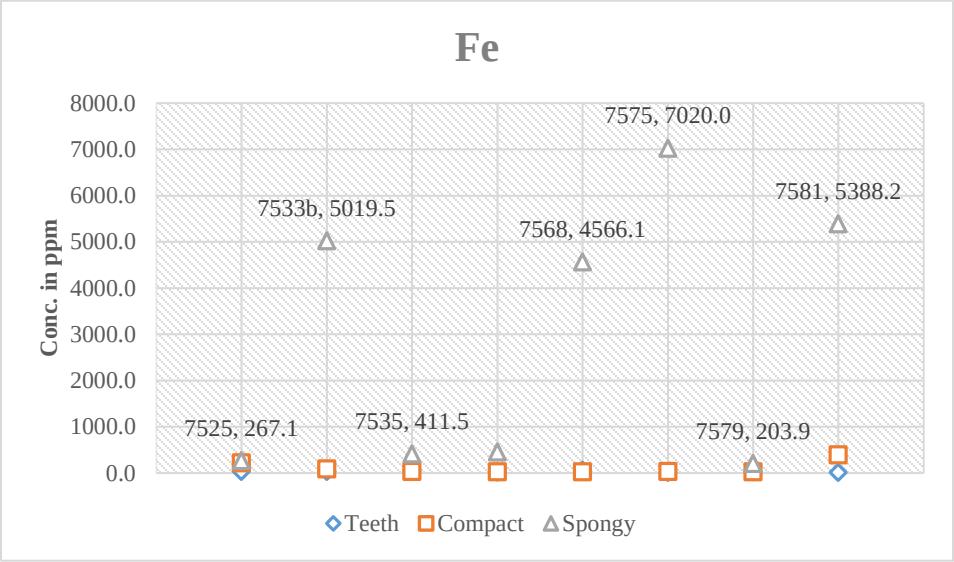

Figure 25: Fe levels in different tissues in different samples

The levels tend to fall down from spongy tissues to compact bones and further diminish in the case of tooth enamel. This can be understood as a case of diagenetic alteration and uptake of these elements through the burial environment.

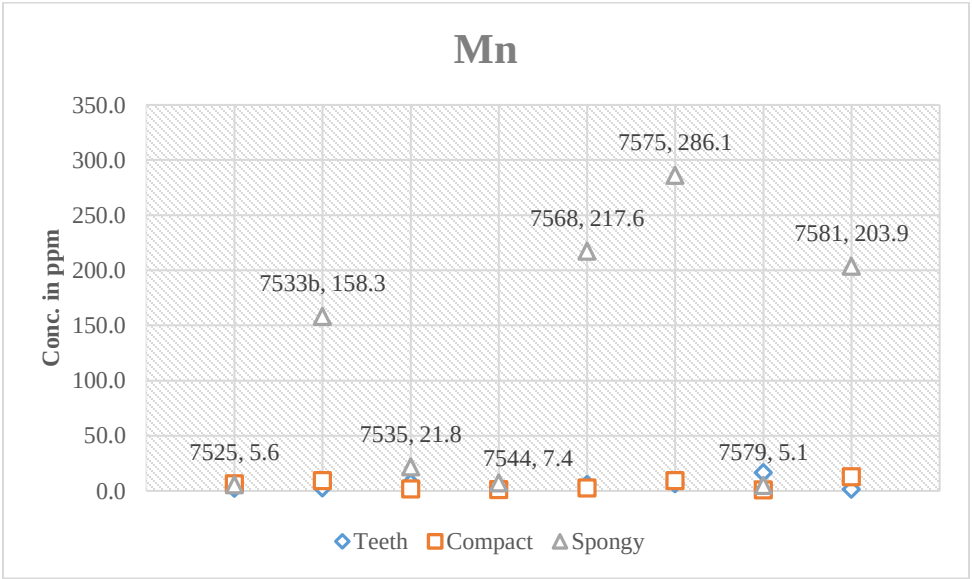

Figure 26: Mn levels in different tissues in different samples

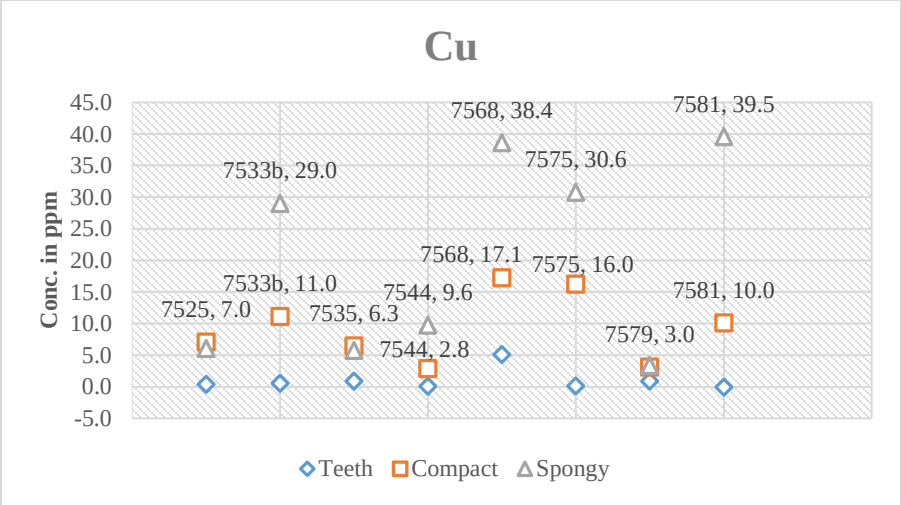

Figure 27: Cu levels in different tissues in different samples

In case of zinc, the levels in both kinds of bone tissues are more or less similar without any significant differences (Figure 28).

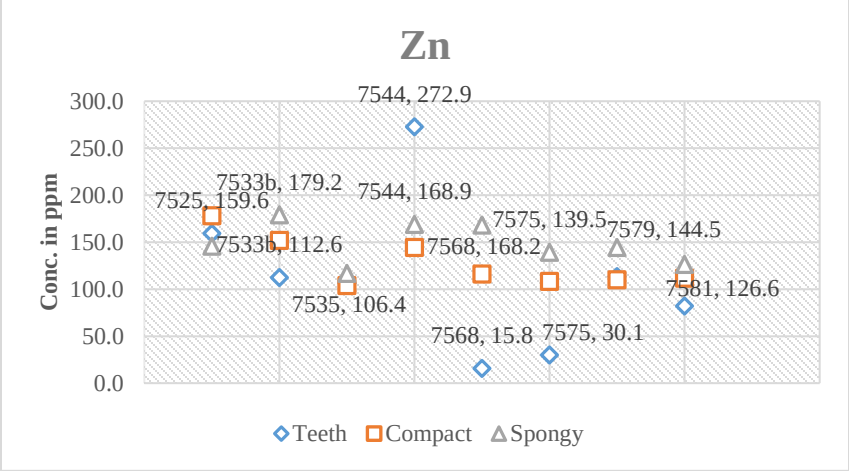

Figure 28: Zn levels in different tissues in different samples

In case of Sr and Ba, the cortical bones display more levels than in the spongy bones (Figure 29, Figure 30). The reasons are not known but it is an important observation nonetheless. The diagenetic uptake of Ba and Sr from the burial soil cannot be completely ruled out and has been known for a long time.

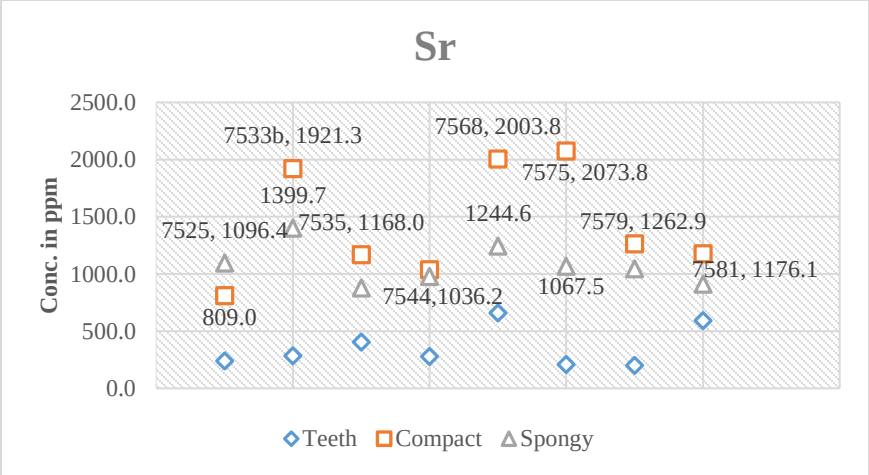

Figure 29: Sr levels in different tissues in different samples

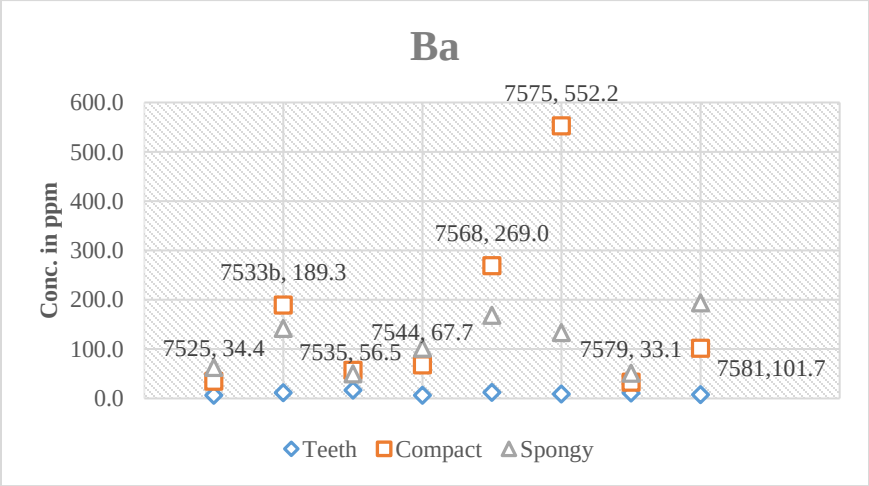

Figure 30: Ba levels in different tissues in different samples

The origin of contamination can be either from the burial ground or somewhere during the preparation of samples in the laboratory. In case of spongy bones with abnormally high concentrations for Pb, Fe, Cu and Mn, they most probably account for post mortem uptake which has not affected the compact bones and tooth enamel to such an extent. It is clear from this study that Pb, Mn, Fe and Cu present in the burial soil from this site are quite capable of penetrating in to the bone tissues more so into the spongy bone tissues and thus are quite enriched in the samples all throughout the spongy bones.

Lead can be both antemortem or post-mortem in terms of its source while zinc with its relatively steadier levels through all the tissues is most probably accumulation by the body while living but some uptake of zinc through the outer layer in contact with the soil has already been discussed by earlier authors (Carvalho, 2004). Zinc is closely regulated by the body as well. Zinc contamination is also very common in the laboratory preparations as well as it might be present in environmental dust, laboratory accessories, handling gloves and so on. All the necessary precautions such as using vinyl gloves and cleaning of all the pipettes and preparation vials and beakers with dilute acid and water were undertaken. The preparation blanks did not display any significant contamination during sample preparation in the laboratory. Nonetheless, Zn contamination cannot be completely ruled out. Even then, Zn does not appear to have been affected by diagenetic uptake to a great extent.

Sr and Ba levels are more ambiguous in their origin. These two elements can be assimilated in vivo in the bioapatite structure of bones as well as some uptake from the soil cannot be excluded.

6.6 One-way ANOVA and Tukey's HSD

In order to understand which TEs and tissues were the most influenced by diagenetic alteration, the elemental result for each TE was treated with a one-way ANOVA at 95%age confidence level, to make a preliminary examination for whether the difference in the concentration among enamel, spongy and compact bones was statistically significant or not (Table 15. Appendix III). The null hypothesis was framed to state that the difference between the mean concentrations for the tooth enamel and the two types of bone tissues is not significant.

Table 17: Example of Tukey's HSD result for strontium.

	Tukey's Honestly Significant Difference			**HSD**			
		critcal q (α, r, dfW)					
	xi-xj	critical q (0.05, 3, 45)	standardized error	95%age conf Interval for μi-μj		Significant at 0.05?	
teeth-compact	-1072.7	3.4275	77.18	-808.2	-1337.3	Yes	
teeth-spongy	-718.7	3.4275	77.18	-454.2	-983.2	Yes	
compact-spongy	354.0	3.4275	77.18	618.6	89.5	Yes	

All the TEs except Zn have shown statistically significant variations in the mean concentrations among the different tissues and the null hypothesis was rejected in their cases (Table 16, Appendix III). This data was then treated with Tukey's HSD in order to find out which tissues had the highest variation between themselves for each element and by how much. The confidence interval was taken at 95%age and 99%age. If interpretation of results from Tukey's HSD is attempted at 95%age confidence level, taking the example of strontium (Table 17), it can be said that tooth enamel and compact bones do not display similar levels of strontium, and we're 95%age confident that a batch of 8 samples of tooth enamel will display 808.19 ppm to 1337.28 ppm less strontium concentration than in compact bones. In a similar manner, it can be said with 95%age confidence that compact bones will display 618.57 ppm to 89.49 ppm more Sr than in spongy bones. All the Tukey's HSD results are given in the table 18, appendix III and can be interpreted similarly. Most of the differences were significant at 99%age confidence level (Table 19).

Table 19: Results for significant difference among elemental concentration between different tissues.

	Tukey's Honestly Significant Difference (HSD) results					
	Pb	Fe	Mn	Cu	Sr	Ba
Teeth	0.26	55.20	5.69	1.02	358.63	9.85
Compact	0.51	165.20	5.65	9.15	1431.37	162.98
Spongy	52.89	4603.48	113.21	20.22	1077.34	112.69
teeth-compact					**	**
teeth-spongy	**	**	**	**	**	*
compact-spongy	**	**	**	**	**	
	*α=95%age	**α=99%age				

Therefore this analysis helps to identify the elements most susceptible to diagenetic changes and mostly in case of trabecular bone, there is a much higher concentration of trace elements as compared to the enamel. Only Sr and Ba are the elements which seem to have significantly higher levels in compact bones compared to teeth enamel. The fact that Zn does not show significant deviations between the different tissues might lead to the conclusion that it is the least affected by diagenetic uptake in the concerned archaeological site or could be the result of a mixture of different biogenic and diagenetic factors as well as contamination.

6.7 Cluster analysis

Cluster analysis was done to group the individuals according to the trace elemental concentration based on compact bone and enamel using elements found to be important for making dietary inferences viz. Ba, Sr, Zn and Mn. It was done to be able to place the samples in possibly distinct diet preference groups. Clusters were also made using Sr and Zn concentrations since they have been reported to be quite useful in classifying the individuals into herbivore, omnivore and carnivore diet preferences.

The cluster reports based on tooth enamel and compact bones are given below (Table 20, Table 21). Bivariate plots were also produced for the clusters between pairs of elements. Examples are displayed in the appendix IV.

Table 20: Cluster analysis based on tooth enamel.

	K-Means Cluster Analysis Report			
Cluster Means			Sample No	Cluster
Variables	Cluster1	Cluster2	7533b	1
Ba	11.1	6.1	7579	1
Sr	391.8	259.3	7525	2
Mn	6.7	2.5	7575	1
Zn	72.9	204.9	7581	1
			7535	1
Count	6	2	7544	2
			7568	1

The cluster analysis based on tooth enamel and compact bones does not match with each other. In case of tooth enamel, the first cluster is very big with six samples and the rest two viz. 7525 and 7544 are in the second cluster. The first cluster with higher values for Ba, Sr and Mn along with low values of Zn might be regarded as consuming relatively more plant food than the second cluster of two samples (Table 20).

However in case of compact bone clustering, the clusters change completely with the second cluster of only two samples but not the same samples as in the previous one (Table 21). In this case, the second cluster with sample number 7575 and 7568, is inclined more towards higher values of Ba, Sr, Mn. Hence the samples 7575 and 7568 are samples which are in a similar cluster

with higher values of Ba, Sr, Mn and lower Zn in both the cases and could be considered positively to be consuming more vegetal components than the rest of the group while 7525 and 7544 are in clusters with lower values for Ba, Sr, Mn, and higher Zn for both cortical and enamel tissues who might had been consuming larger portion of meat components in their diet.

Table 21: Cluster analysis based on compact bones.

Cluster Means			**Sample no.**	**Cluster**
Variables	Cluster1	Cluster2	7533b	1
Ba	80.4	410.6	7579	1
Sr	1228.9	2038.8	7525	1
Mn	5.5	6.1	7575	2
Zn	126.9	107.0	7581	1
			7535	1
Count	6	2	7544	1
			7568	2

In case of trabecular bones, the values were found to be more susceptible to diagenetic changes and therefore have not been used for cluster analysis. In conclusion, the picture might be clearer with more samples from the same site for further study. Also in all the samples and the clusters, female and male elemental data conspicuously resemble each other, and therefore similar dietary intakes for both the genders can be hypothesized within the dietary groups. The gender differences might amplify though on the examination of more sample.

Cluster analysis using Sr and Zn was also made based on compact bone results since compact bones were found more reliable in case of Sr levels (Table 22). Theoretically, higher Sr content should be supported by lower Zn level. Therefore Sr and Zn can be related to define the trophic level of individuals and their inclination towards a more herbivore/carnivore diet. In this study, all the lowest Zn level and highest Sr samples belong to the cloister courtyard while the highest Zn and lowest Sr levels were found from the church nave samples while three of the samples fall at intermediate range. The individuals 7525 and 7544 in cluster 3 from the church site are thus conjectured to have higher proportion of meat proteins and lower plant food than those from the cluster 1. Cluster 2 falls between cluster 1 and cluster 3 as far as the Zn and Sr levels are concerned.

All the individuals belonging to cluster 1 and 2 are from the cloister courtyard except 7535 (Figure 30).

Table 22: Cluster analysis based on Sr and Zn levels.

Cluster Means using cortical bones						
Variables	Cluster1		Cluster2		Cluster3	
Sr	1999.6	7533b	1202.3	7579	922.6	7525
Zn	119.4	7575	103.7	7581	153.2	7544
Count	3	7568	3	7535	2	

It is clear from all the above observations that correlational studies between different TEs can help to find similarities among elements and their association in case of biogenic or diagenetic pathways. They can even indicate synergistic or antagonistic relationships between elements which have not been discussed much even in previously published literature. Such studies have been undertaken in case of other mammals such as foxes and domestic dogs (Lanocha et al., 2012; Budis et al., 2013; Budis et al., 2015) using different kinds of bone tissues and cartilage but not in case of human remains from archaeological contexts.

It is not correct to attribute all the correlations directly to biogenic or diagenetic relations. While correlations such as those of Fe with Mn, Cu and Pb in case of spongy bones can be attributed to diagenetic uptake there are many relationships which might be simply antagonistic or synergistic in nature. In the present work, the positive correlations of Zn with Sr in trabecular bones and of Ba with Sr and Cu in cortical bones can be considered as synergistic. Copper displayed a very strong synergistic association with barium in trabecular bones.

On the other hand, antagonistic relations were found in case of P with Cu, Ba, Mn and Fe as well as in case of Ca with Ba, Cu and Fe for trabecular bones. Positive correlations in case of trabecular bones including Mn, Cu, Fe and Pb might also be synergistic but are most probably due to association in diagenetic pathways.

To summarize this section, many correlations have been found among the elements studied. Elemental levels did not display any significant differences among the genders but in case of the two different burial areas viz. the church and the cloister, significant differences were found in the Pb content of the enamel as well in the strontium and Sr/Zn ratios for the cortical bones. Pb, Mn,

Cu and Fe levels in trabecular bones were much higher than the rest only in the case of four individuals which most probably is the consequence of the area of extraction of the sample. Sr and Ba displayed much higher levels in cortical bones the reasons for which is not clear. Zn was the only TE that didn't show any significant differences in the levels between all the tissues. The cluster analysis does not display any gender based groupings. Nevertheless, the samples from the church seem to belong to a higher meat consuming group as compared to those from the cloister.

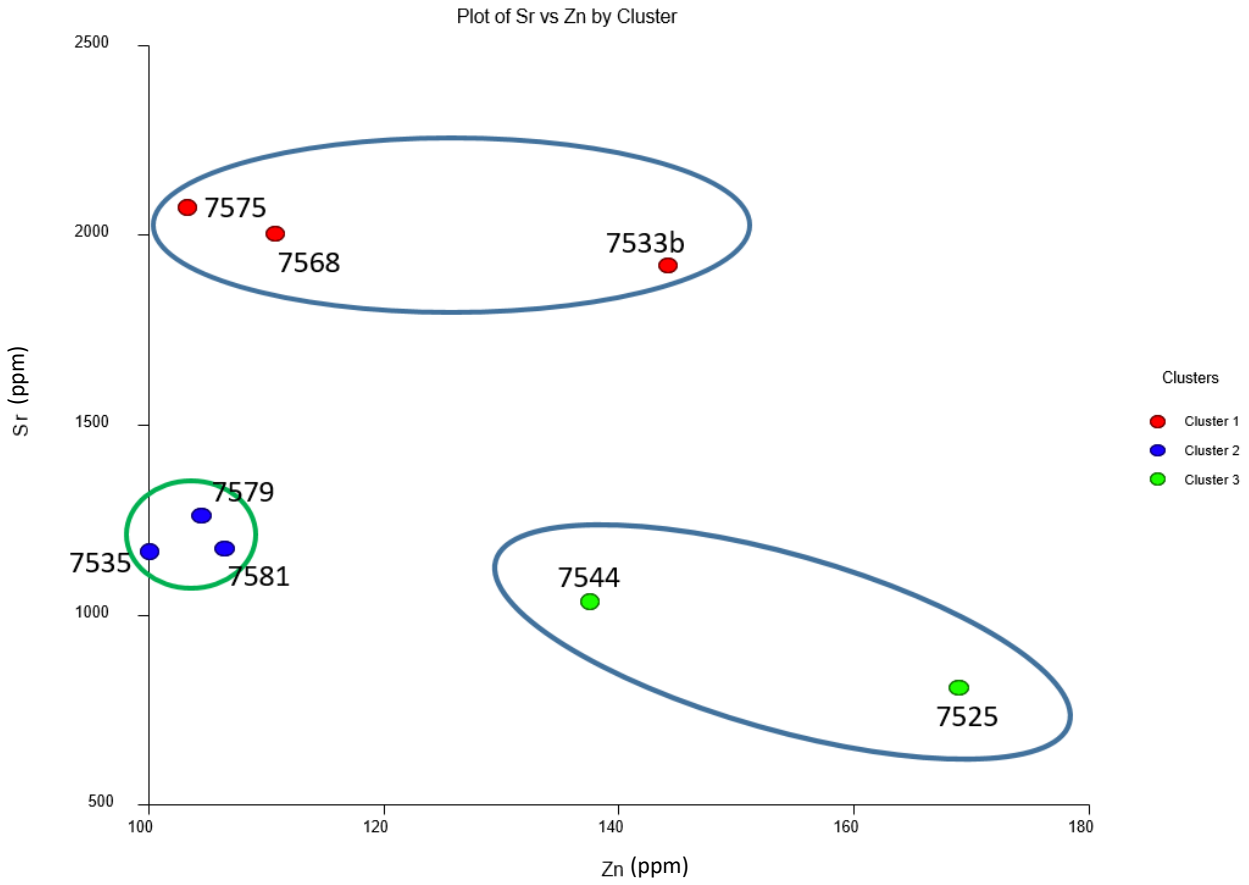

Figure 31: Clusters based on Sr and Zn levels (ppm) in cortical bones

6.8 Discussions on diet and microwear results

Strontium is considered to be gathered from plant food but it further complicates the situation given the fact that seawater and marine products are also highly rich in Sr (Leach et al., 2003). It can also be contributed due to the Sr content of the carbonate soil. The Sr concentration in the compact bone tissues ranged between 809 to 2074 ppm. This high concentration of Sr in the bones

can be due to either the Sr content of the soil or due to consumption of Sr rich food stuff by the individuals under study which is not clear with this analysis. Sr is used in the ancient diet research to find whether the community relies more on inland nutrition or on marine mode of nutrition (Connor & Slaughter, 1984). In this case, the data of the present study needs to be compared with data from archaeological sites closer to the sea to be able to compare the Sr levels properly and make inferences on the mode of nutrition. This might help to understand whether the Sr levels are related more with plant foods or with marine type of nutrition.

The results need to be supported by the study of Br levels which can make the picture clearer. Bromine is an element which is higher in diets rich in fish, molluscs, crustaceans etc. along with strontium. The presence or lack of marine food can be confirmed by analysing Br content in the future.

The attempt at understanding the dietary differences among the population usually makes use of elements such as Sr and Ba which are well known indicators of food intake. The values of the log Ba/Sr in the bones usually is a good indicator of a mixed menu of the individuals. The lower the value, the higher is considered the percentage of the marine food in the diet (Allmäe et al., 2012) although it is not very helpful to distinguish between freshwater fishes or marine fishes (Burton & Price, 1990). In case of most of the individuals, these values are ranging between -1.58 to -1.00 except in case of one male and one female, the value rises higher than usual in case of male individual 7568 who has a value of -0.87 which might still indicate a mix of meat proteins or marine food but in case of the female 7575 this value rises to -0.57 which shows that she has highest log Ba/Sr value in all the samples and probably consumed much more inland food than marine food like the rest of the individuals. Then again the values of log Ba/Sr cannot be directly compared with literature values because they might vary greatly among different sites with different vegetation and also cannot exactly distinguish between fresh water and marine produce.

The use of logarithm of Ba/Sr has been suggested by some scientists in order to differentiate between terrestrial protein and food crops (inland nutritional mode) and coastal agriculture and marine proteins (marine nutritional mode) (Burton & Price, 1990). Values equal to or higher than -0.40 point to an inland mode of nutrition while those smaller than -1.40 denote a more marine type of nutrition for the ancient community (Allmäe et al., 2012). Only one of the value is smaller

than -1.40 for individual 7579 while all the rest fall between -0.40 to -1.40. It might be therefore, the result of personal metabolism/food preferences for this individual.

If Burton & Price (1990) is taken as a reference (Figure 32), then the values of log Ba/Sr for the current study fall within the ranges of terrestrial and freshwater food components. Given that all the values fall between -0.57 to -1.58 (Figure 33), a terrestrial diet along with freshwater resources is speculated for the individuals under study. Nonetheless, log Ba/Sr values need to be evaluated for coastal communities in this area to be able to make any concrete conclusions.

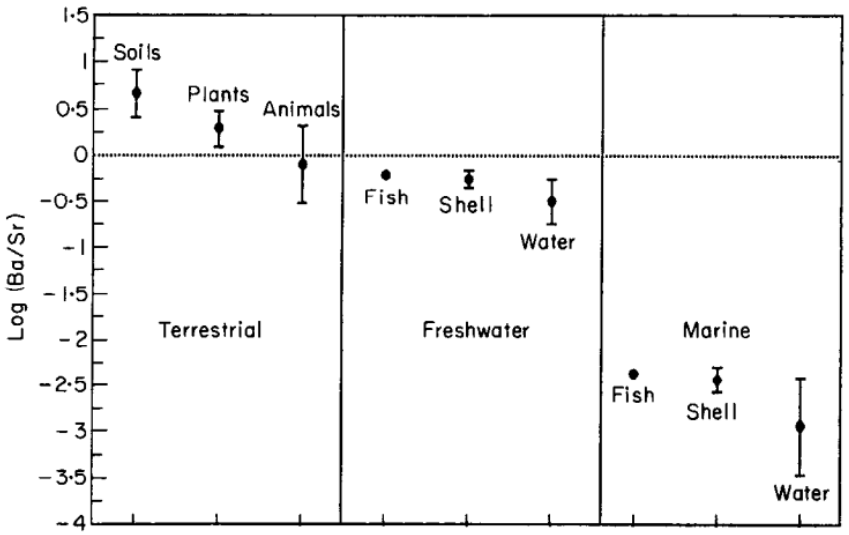

Figure 32: Mean log (Ba/Sr) values from published data for water and terrestrial samples (Burton & Price, 1990)

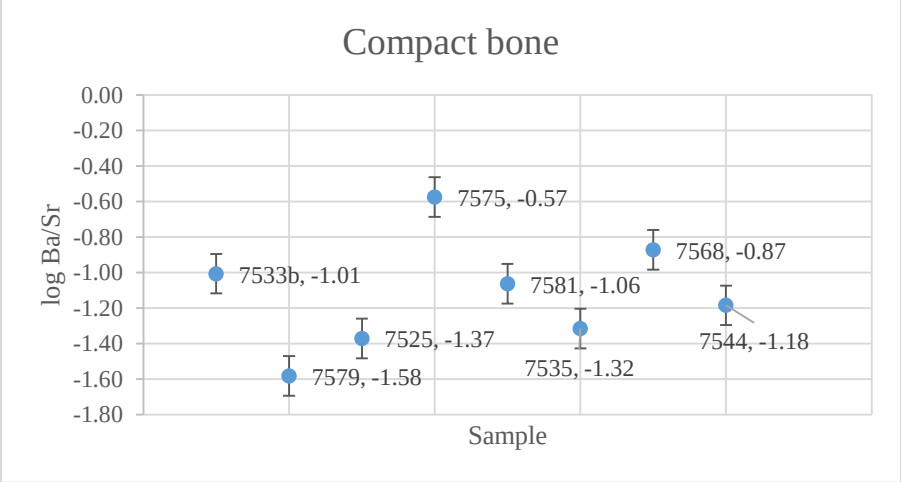

Figure 33: Log Ba/Sr values for all the samples

Notably, the log Ba/Sr values are much more variable in case of women than in case of males with a range of 1.00 and a variance of 0.19 while in case of males the range is only 0.44 and the variance is about 0.04 but the differences are not statistically significant. It may point towards dissimilar origin of the women (the distance of living place from a marine food source or the accessibility to marine food) and also the differences in the composition of their food and dietary habits. A lower log Ba/Sr ratio thus can point towards a greater percentage of marine food (or freshwater resources) in their choice of menu. No significant differences were found also between the cloister and church samples.

Interestingly the female 7575 with the highest value of log Ba/Sr also displays the highest level of copper of about 16.3 ppm which calls for attention. Higher the level of Cu, higher is the fraction of meat and fish in the person's diet. In case of males also, the highest value of Cu i.e. 17.2 ppm is associated with the highest value of log Ba/Sr. On further analysis, it was found that Cu levels are strongly correlated with log Ba/Sr values (Figure 34). The exact explanation for this strong correlation is not clear.

The concentrations of Ba and Sr can also be compared to that of Ca in order to make inferences about the trophic level of the diet of these individuals (Figure 35). Relatively high ratios of Ba and Sr with Ca or in other words high levels of Ba and Sr in the skeletal tissues signal towards a diet based on plants (Burton, 1996).

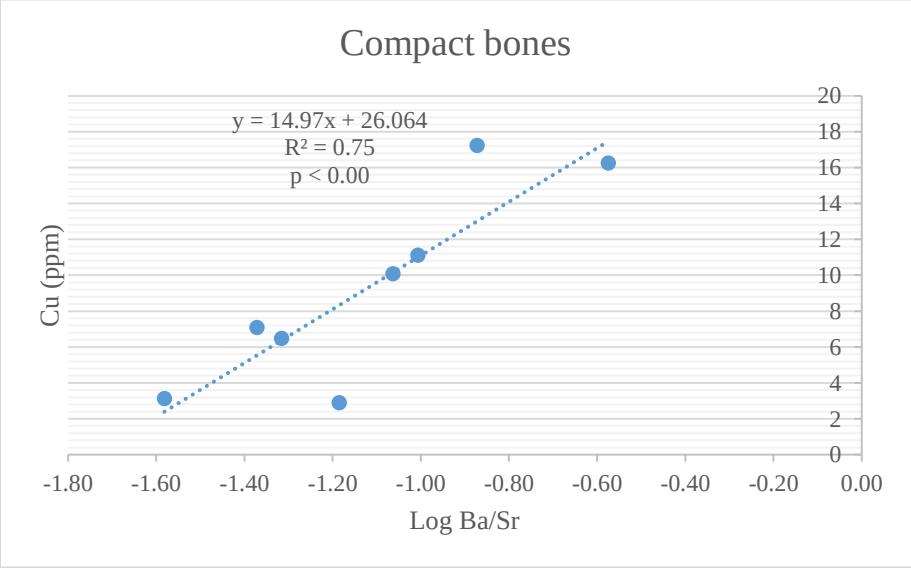

Figure 34: Correlation between Cu and log Ba/Sr values

Inversely, if the diet is composed of more fraction of meat proteins and marine components, it will reduce the value of both the ratios and also the ratio of Ba to Sr which has already been discussed (Burton & Price, 1990). Strontium levels in the teeth and bones of the people who eat more meat is lesser than those who have a more herbivore diet.

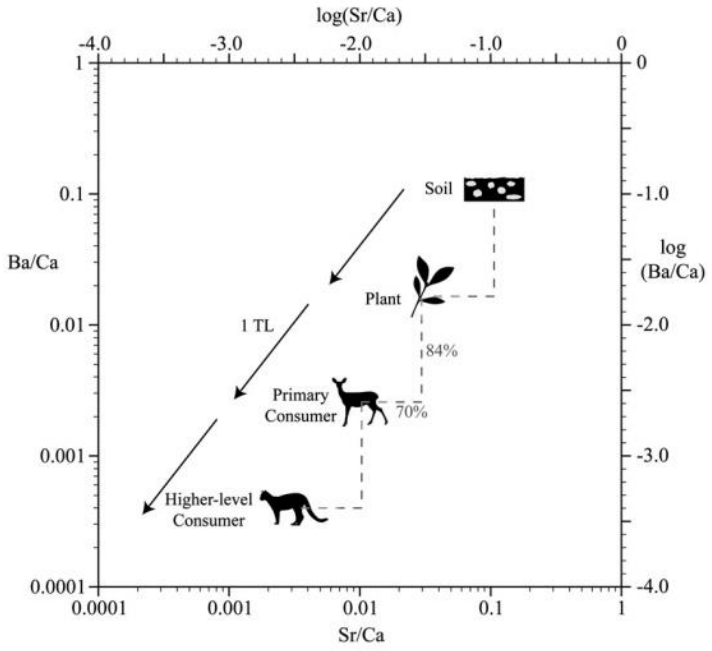

Figure 35: Trophic level differentiation based on log Ba/Ca (ppm/ppm) and log Sr/Ca (ppm/ppm) values (Peek & Clementz, 2012)

Burton (2007), has found great dissimilarities in the Ba levels in bones from inland archaeological site and those from marine sites. This difference was not so large in case of Sr due to the solubility of Sr in the presence of sulphate ions rich conditions in the form of Strontium Sulphate. On the other hand Barium Sulphate or Barite (BaSO4) is quite insoluble and thus is expelled from the environment. This lowers the ratio of Ba/Ca in case of marine food habits (Burton, 2007). The distinction becomes more difficult since low Ba/Ca ratios are exhibited in both marine and carnivorous diets. On consumption of any food matter, the Sr/Ca and Ba/Ca ratios reduce further than that in the food source due to the preference of every animal or human to intake more Ca rather than Sr or Ba, which is the foremost constituent of the hydroxyapatite of bones and teeth. But this effect won't be properly showcased in case of a menu with different kinds of food materials. At that point, the correspondence becomes more complex. It is properly showcase only if the food has one type of constituent such as only pure plant or pure meat based diets. For example

even ingestion of a food component with low Ca amount can reduce the ratios due to the favored absorption of Ca by the body. The log Ba/Ca and log Sr/Ca (ppm/ppm) values from compact bone (for reasons already mentioned) can be compared with literature values to understand better the food components of the diet of the individuals under study. Peek & Clementz (2012) have gathered log Ba/Ca and log Sr/Ca values for major food groups (Figure 36).

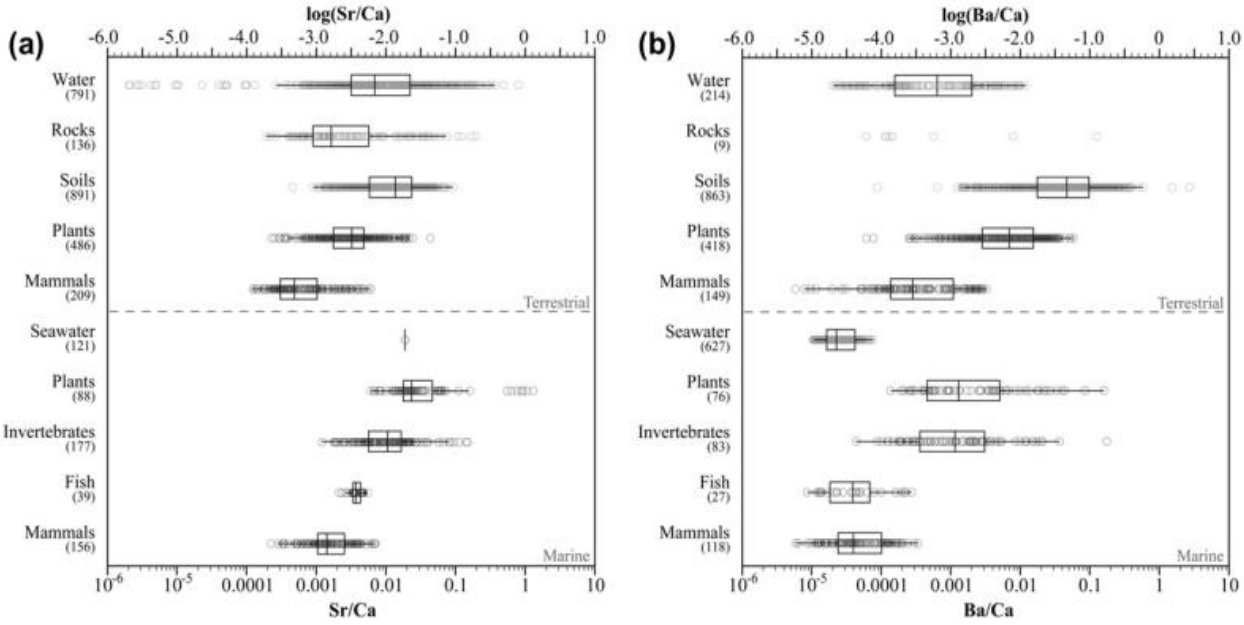

Figure 36: Sr/Ca and Ba/Ca ratios for natural samples (Peek & Clementz, 2012)

On comparing the values with those given in literature, all the samples fall between primary consumers and higher level consumers in the trophic level. All the samples from the church nave display lower values for both the ratios possibly at a higher trophic level than the rest of the samples (Figure 37).

The levels can also be compared to those in major food groups given the fact that the levels of Sr and Ba are always lower in the consumer as compared to the source. For such calculations, the Sr/Ca and Ba/Ca ratio is also calculated for the diet sources and is compared with the values from the consumer. This relative decrease in the ratios of Ba and Sr in relation to Ca, between the consumer's tissues and the diet, is quantified using observed ratios (OR) (Comar et al., 1957) also known as Trophic Transfer Factor (TTF) (DeForest et al., 2007).

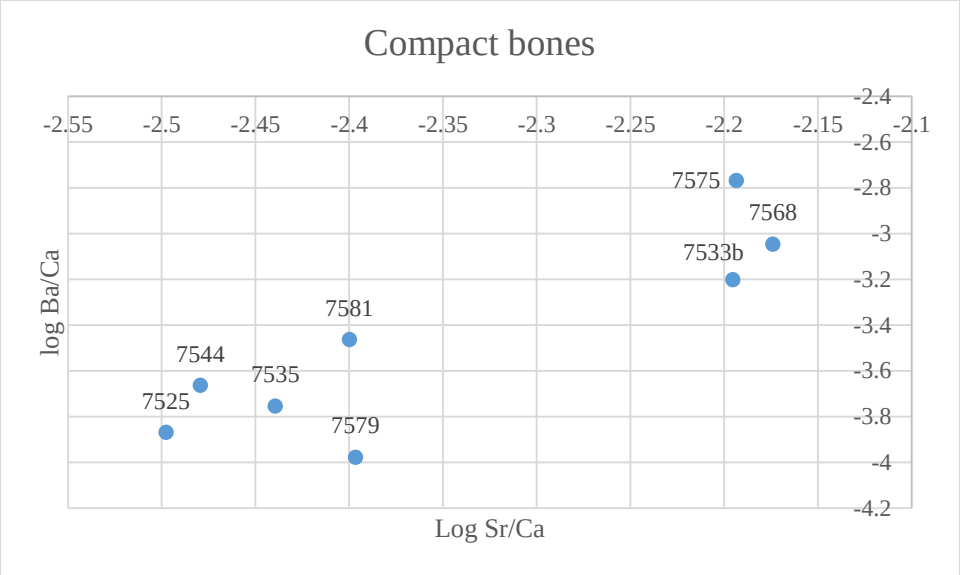

Figure 37: log Ba/Ca (ppm/ppm) and log Sr/Ca (ppm/ppm) for the compact bones of the samples

The use of Ba, Sr and Ca is not very straightforward to reconstruct diet. Apart from this, the amount of Ca intake by any organism depends on the type of edible material and its particular Ca metabolism (Reynard et al., 2011). There are plants that have high concentrations of Ca such as seeds, nuts etc. since it is required by the plants as well for their development. Then there are grains such as wheat and others that do not have high Ca content (Pharswan & Farswan, 2011). There even exist plants which contain more than ten times the Sr and Ca found in meat and clearly make a huge effect on these ratios (Burton & Price, 2002). At the end of the day, the straight correlation between these alkaline elements and the food habits is further disturbed by other factors such as individual variability of metabolism, diet with a mixture of different kinds of food, the environmental conditions of the locality, the heterogeneous nature of vegetal diets and most of all diagenetic pollution (Burton & Price, 2002).

Thus, these ratios do not have a linear correspondence with plant/meat proportions in food and vary greatly among individuals as well (Burton, 2007). The concentrations of Ca, Sr and Ba varies from location to location and such geographic variations make it difficult to be able to compare food habit reconstructions among different sites (Burton et al., 2003). Some scientists have even used Ba/Sr ratios for mobility studies rather than the reconstruction of palaeodiets (Arnay et al., 2009; Brügmann et al., 2012).

Even among plant food, the variability of ratio of Sr to Ca is very high between green leafy vegetables, corn, nuts and others. Hence instead of being able to directly point out the proportions of meat and vegetal components in the diet, these ratios point more towards the status of grazing and browsing in that time (Burton & Price, 2002). Even marine food or meat in cases might have Sr/Ca ratio comparable to a plant with low Ca content such as wheat (Ezzo et al., 1995). For different kinds of animals or humans, the same plants will leave different TE concentrations based on the difference in their absorption in the skeletal tissues of different organisms.

Zn level has been known to display differences in the trophic level by the variability in its concentration in the bones of humans as well as animals (e.g., Rheingold et al., 1983; Schutkowski, 1995; Grupe, 1998) which in turn are indicative of distinct dietary habits. Nonetheless Zn is still an enigma in many ways when it's comes to the knowledge of metabolic reactions through the bones and therefore researchers are not of one opinion when it comes to the importance of differences in the Zn (and Cu) contents in the bones (Ezzo, 1994a, b.) As a result of the ambiguity in the data, the results from these elements are taken to be as preliminary results along with a struggle to establish their acceptability. Zinc levels can exist alongside variable Ca supply levels. Most part of food components which have already been discussed such as vegetal parts, except milk, is enriched in Zn. Compounds such as cellulose, hemicellulose and phytate impede the rate of absorption of Zn by the intestines which is also known as its bioavailability (Bender, 1993). These compounds are mostly found in plant food and put a restrain on the resorption of Zn.

Thus a better bioavailability of zinc for an individual will automatically lead to higher zinc levels for them. Most probably Zn resorption in such individuals have been augmented with the complementary supply of meat proteins, milk products and legumes in the food. (Burton & Wright, 1995). The concentration of Zn in an individual's bones with an omnivore diet usually ranges between 50–826 ppm (Allmäe et al., 2012).

Palaeodiet studies have also employed Zn/Ca relations to identify higher or lower protein intake. Zn/Ca reference values >0.5 equivalent to a diet rich in protein and <0.35 equivalent to a diet poor in protein were used from previous literature and have been compared for the current data (Gallello et al., 2015). While two of the individuals seem to be in the rich in protein diet group (7533b & 7525), three samples i.e. namely 7579, 7575 and 7535 edge more towards the poor diet group. The rest of the samples fall in the intermediate levels (Figure 38).

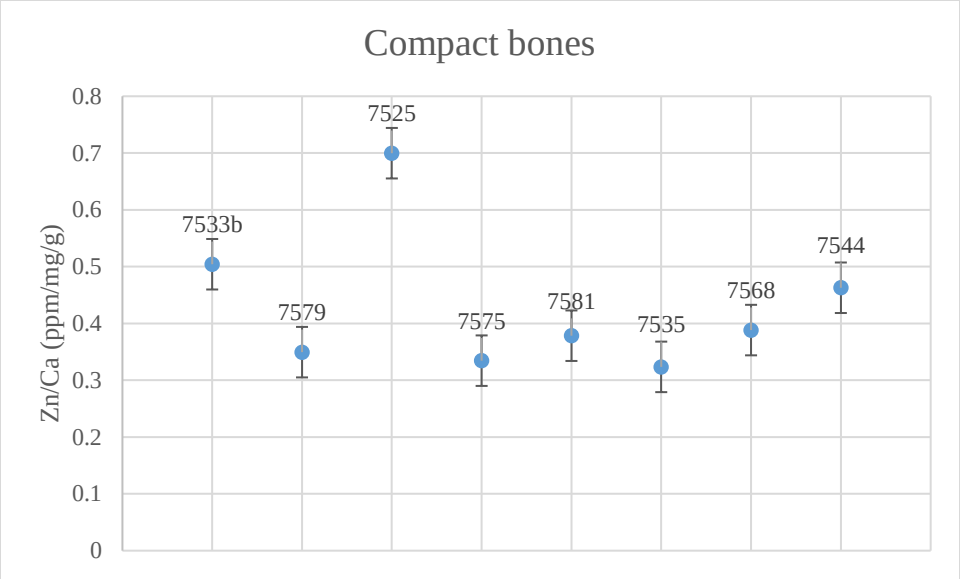

Figure 38: Zn/Ca (ppm/mg/g) values for the compact bones of the samples

Although it is common knowledge that plants are the main source of Mn content in the diet, the level of Mn in the plant is directly influenced by the content of Mn in the soil where they are grown. The results of Mn are in accordance among themselves theoretically because Mn is better absorbed by the body in the presence of animal protein in the diet and not only because of the presence of plant foods in the diet (Kies, 1987).

Copper and iron have not been too helpful in understanding diet. This is probably also due to the dearth of literature data which could help to interpret the role of these elements in diet reconstruction. Furthermore, these two are closely regulated by the body and display antagonistic as well as synergistic relationships with each other in the body.

There is a great variability in the concentration levels of all the TEs discussed here in case of different individuals and tissues. Making inferences on general menu of the community is more complicated rather than comparing the differences between different individuals' diet based on their variable elemental intake and assimilation.

The diet of the individuals from San Pablo, has been interpreted from the TE data to be a diet mixed of foodstuff and meat proteins or fish. But the consumption of marine products cannot be

proved or disproved. The ratio of vegetal and meat components in the food vary from person to person.

6.8.1 Comparison with microwear analysis results

The buccal microwear analysis results are additional information which is being gathered about the human remains from San Pablo by some scholars from the Universidade de Burgos. At the time of writing the thesis, only three individuals 7533b, 7544 and 7565 out of all the samples had been studied. They have been classified as carnivorous hunter gatherers with a mixed diet including both meat and vegetarian food with a fair meat component according to the results of microwear analysis (Figure 39, Figure 40). This diet even continues to the contemporary times as well in this area (Zuriñe Sanchez Puente, personal communication).

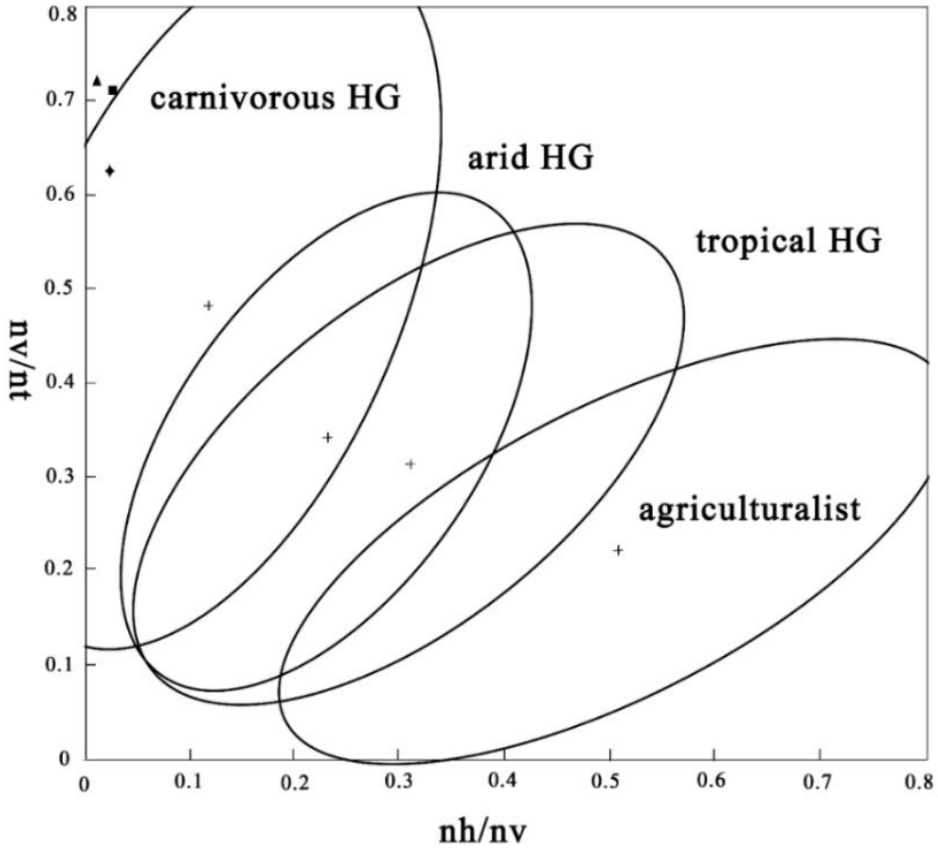

Figure 39: Plot of NH/NT index vs NV/NT index for four dietary groups and the three specimen. Black lines represent the 95%age equiprobability ellipses of each dietary group and crosses are the centroid of these ellipse (Zuriñe Sanchez Puente, personal communication)

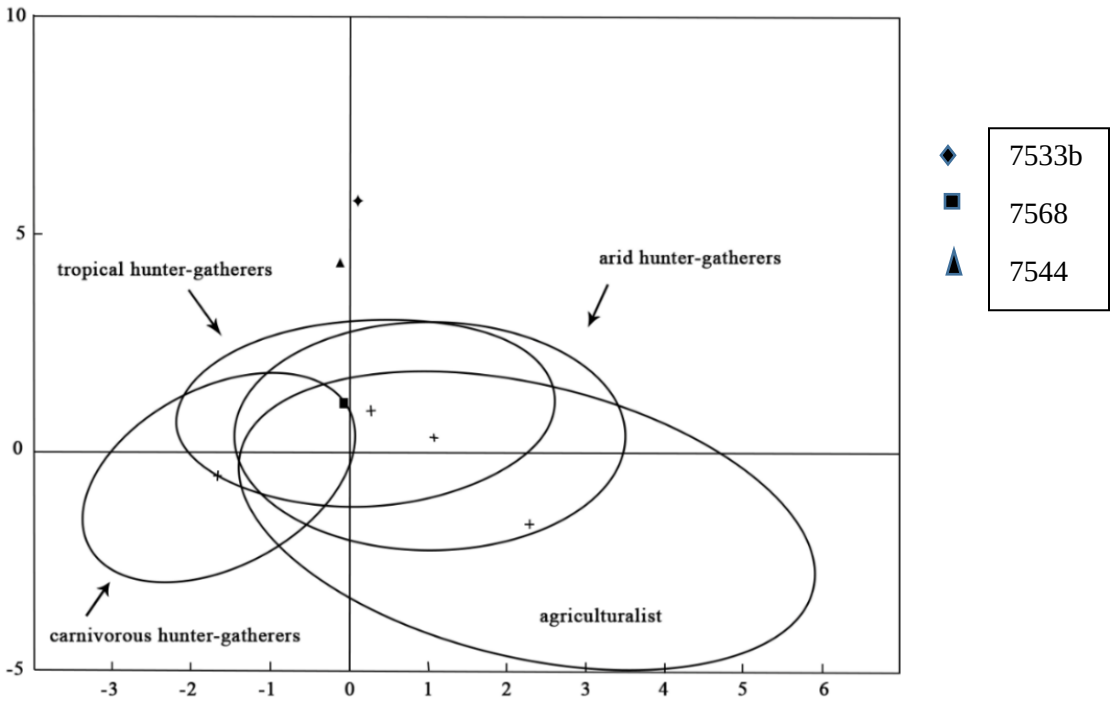

Figure 40: Discriminant function analysis of four dietary groups based on microwear measurements taken on the buccal surface of the three specimen. Black lines represent the 95%age equiprobability ellipses of each dietary group and crosses are the centroid of each ellipse (Zuriñe Sanchez Puente, personal communication)

The overall diet of the middle age community from San Pablo, in conclusion, can be reconstructed as a mixed diet of plant food and meat and fish with possible inclusion of seafood or marine products. What seems interesting is that women might have been consuming lesser meat components than their male counterparts, even though the difference between the sexes was not found to be significant. This gender difference further needs to be studied by analysing more samples from the same site(s). The same interpretation has been given about men consuming more meat than women during middle ages in Palencia by Perez-Perez et al. (1994) on his study using buccal microwear analysis on 99 samples from La Olmeda. Palencia is about 100kms away from Burgos region and the communities living in these sites in middle ages most probably had the same subsistence patterns and modes of nutrition.

There is definitely a significant difference in the food preferences of the samples buried in the church nave and those buried in the cloister courtyard which seems to indicate a bigger percentage of meat proteins in the menu of the church samples. This might indicate a difference in status or even changes through time in diet preferences.

7

Conclusion

This study on TEs shows a great variability in the results which have been discussed in detail. By controlling the possibilities of contamination, cleaning, limiting the area of extraction and homogenizing the sample type, more reliable results can be gathered. Using regreassion analysis between different elements, many antagonistic and synergistic associations came to light. Some of these could be easily pinned onto diagenetic/metabolic associations while the rest are not fully understood. Correlation between the Ba/Ca and Sr/Ca ratios helped to test the reliability of results from all the three kinds of tissues, of which, cortical bones showed the strongest correlation. Thus, the cortical bone TE results were used to make inferences on the diet.

The diet of the middle age community from the archaeological site of San Pablo monastery has been interpreted to be a mixed diet of plant food and meat components in their menu. Even to this date, the diet in this area is mixed which has higher meat components than vegetal components inclined towards a more carnivorous diet. It is no wonder that the middle age communities might be having a similar diet.

The men might be having more access to meat than women but this fact needs to be further investigated by analysing more samples from this site. This result was in synch with that gathered from microwear analysis and other literature previously published. Differences between different age groups and information about weaning might also be gathered in future by examining specimen from different age groups. Most probably the results indicate individual food choices rather than a general dietary pattern of the community.

Most certainly, the individuals buried in the church nave had a diet more rich in meat proteins than those buried in the cloister due to the significant differences found in the levels of Sr and Sr/Zn. Moreover, they have displayed higher Pb contamination in the enamel. These differences are worth looking into with analysis of more samples. The reasons for this might be chronological differences or differences pertaining to social class. To be able to pin point the reasons, it is necessary to analyse more samples belonging to different time periods from the same burial area or vice versa.

As far as diagenesis is concerned, Fe, Cu, Mn and Pb levels in trabecular bones indicate post mortem uptake of these elements especially through the inner surface of the femur shaft. The situation in case of Ba and Sr is not very clear given the high concentrations in compact bones rather than in spongy bones. Zn levels didn't give any clear evidence of diagenetic uptake given the fact that there wasn't significant differences in the levels through all the tissues.

Despite all the extensive knowledge that can be gathered from the analysis of TEs, one can never be fully confident about the conclusions when it comes to the reconstruction of palaeodiet since the concentrations of TEs are modified due to the environmental and temporal factors. The unique and complicated biogenic and diagenetic properties of every tissue in every individual further obscures the research. Additionally, different plant species and different individuals have their own level of TE absorption even if they belong to the same archaeological setting. As a result, there are certain possibilities of making mistakes when it comes to the interpretation of the chemical composition of archaeological bones and tissues. The physical, chemical and biological factors cumulatively act on the human tissues and their chemical composition in vivo as well as post mortem. This is the reason why it is important to identify tissues from which biogenic information could be extracted efficiently and unfailingly.

Until a working model has been achieved for the quantification of post-mortem alterations such as diagenesis or other taphonomic changes, nothing could be inferred with absolute surety. In recent years, thus, the focus of study has shifted from diet palaeodiet reconstruction towards the investigation into post mortem alterations as well as the construction of models to be able to quantify the extent of these changes. This study tries to study this aspect as well and the comparison between trabecular bones, cortical bones and tooth enamel provides quantification of diagenetic changes to some extent. Furthermore, the elements which are most affected by diagenetic uptake need to be singled out in order to establish their reliability. In the study at hand, zinc concentrations have been found to be the least affected by diagenetic uptake or might have been affected in all tissues to the same degree. Many other factors might be at work at the same time. Additionally, it is clear that each skeletal part has a different reaction to diagenesis even in the same area of burial.

Even though the samples were thoroughly cleaned, the contamination of teeth due to soil constituents cannot be completely ruled out. The collection of sample was undertaken with great care so as to avoid contamination chances as much as possible and yet it is quite probable that the

content of Pb, Sr and Zn is much more in the samples than has been found out from the observation since they have a high chemical affinity to replace P and Ca in the crystal of hydroxyapatite (Stevens & Lowe, 1997; Bowen, 1979). The soil composition of the site is important to be reported as well in more detail to be able to quantify diagenesis better. This is not possible in the current case where the site is not available for such analysis in the present time.

This is a new approach to use trabecular bones for the quantification of the extent of diagenesis. This can be further extended into the comparison between more tissues such as dentin and cementum from the teeth to dig deeper into diagenetic changes using the same methods as in the present work. It also records episodes of growth and development for every individuals' life time as was seen in case of first molar from one sample. Enamel might be more resistant to diagenesis but surely is not immune to it.

The grave goods and treatment of the funeral remains in that time are also a key element which can extend the exploration into the social structure, stratification and socio-economic status corroborated with the dietary habits of different groups. Regardless of the broad investigation using statistical tools, there are still unsolved questions about mineralization processes of teeth and bones, the pathways for intake of trace elements both biogenic and diagenetic and many antagonistic and synergistic relationships. The results from such studies therefore should essentially be corroborated with secondary evidences such as microwear studies used in this work which can complement the outcomes. This examination is only a preliminary approach into the human remains from San Pablo using archaeometry. Although, there haven't been such explorations in this site, the present attempt surely gives a positive impression of the potential insight that could be gained from further probes. While a few of these queries may give the impression of being redundant, they set the stage for many impending investigations.

7.1 Future directions

While the results achieved might be considered satisfactory there were quite a few shortcomings regarding the overall investigation method and the scope. These deficiencies have been identified by the author and can be overcome in consecutive attempts at explorations. Additionally, no single work is all-encompassing and as such the study at hand also leaves possibility for advance inquiries on the remains from this vastly ignored site.

Numerous prospects have arisen in concordance with TE analysis. Advances are being made every day to delve even deeper into interpreting the past environmental conditions and how humans adapted to their immediate surroundings. Histological analysis along with Scanning Electron Microscopy to pin point the concentrations of TEs in different regions inside teeth and bone tissues is the next step in this series of investigation. Thin sections of bones and teeth provide a useful support to TE analysis in order to specify the locations of high concentrations of the elements under study which can therefore be valuable to distinguish between biogenetic and diagenetic accumulation. It can also help to reconstruct the pathways of amassing of elemental concentrations in different kinds of tissues.

Elemental signatures and even isotopic signatures are retrievable from archaeological remains such as biogenic apatite present in enamel and bone tissues (Koch et al., 1997, Hoppe et al., 2003). Isotopic studies nowadays, can differentiate between people and even fauna of local origin and non-local origin, provenance artefacts, migrants, building material, foodstuff, track the level of residential mobility patterns in the ancient society (palaeomobility) regarding both humans and animals, health and forensics, imperial strategies, reconstruction of palaeodiets, colonization, trade, exchange, cultural change, ecological shifts and can answer many other pressing questions. Strontium isotopes and lead isotopes have already found a lot of use in such studies and is proposed here as a future avenue for examining the remains from San Pablo monastery.

This diet reconstruction can be complemented by stable isotope ratio studies using carbon, nitrogen and others to achieve a deeper level of comprehension. Recent studies have even utilized iron and copper isotopes to find sexual differences among the past population. 56Fe/54Fe and 65Cu/63Cu ratio is not widely documented yet in archaeological remains and has been found to preserve sex differences (Jaouen et al., 2012). Fe and Cu also give indication towards ancient diseases. With the specimen gender already established in case of San Pablo remains such a study could help to design a method of sex determination using Cu and Fe stable isotopes that can be used in case of sites from where enough material is not retrieved to be able to determine the sex by other methods such as DNA analysis or pelvic morphology. It needs to be tested for different kinds of tissues as well and has a great potential as a sexing tool.

Later studies can be refined using more sophisticated equipment as well as more appropriate standards such as bone meal Standard Reference Material (SRM) 1486 in bone matrix developed

by National Institute for Standards and Technology (NIST, USA) which was unavailable for the present work. A new opportunity of research in the biological context is the use of new non-conventional isotopes of heavy essential elements (Costas-Rodriguez et al., 2014) some of which have not yet been studied with a biological viewpoint. For example bioessential elements like Silicon (Si) might reserve a great potential for palaeoenvironmental and palaeobiological relevance (Schwarz & Milne, 1972) which exists in notable quantities in the human body (Iyengar, 1998). The quantification of REEs such as Y and Zr and radiological elements such as U, Th, Cs and others can also be undertaken in order to create models for diagenesis level detection and dating.

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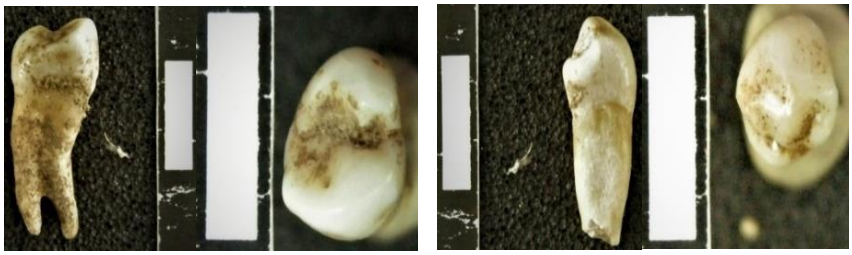
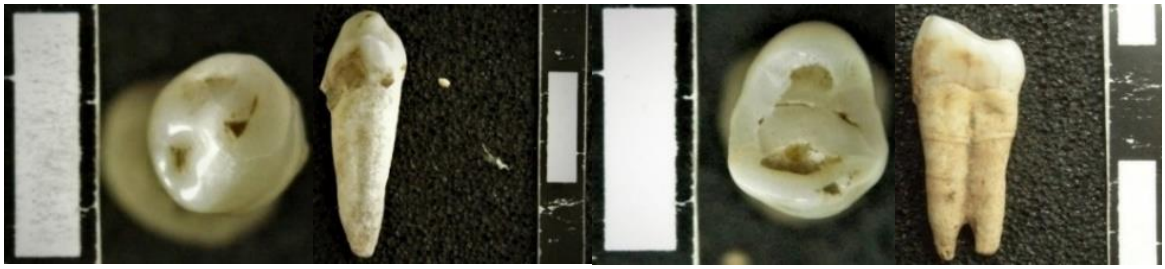
Appendix I



a b

Figure 41: Occlusal and buccal views of a) SP 7533b, b) SP 7525

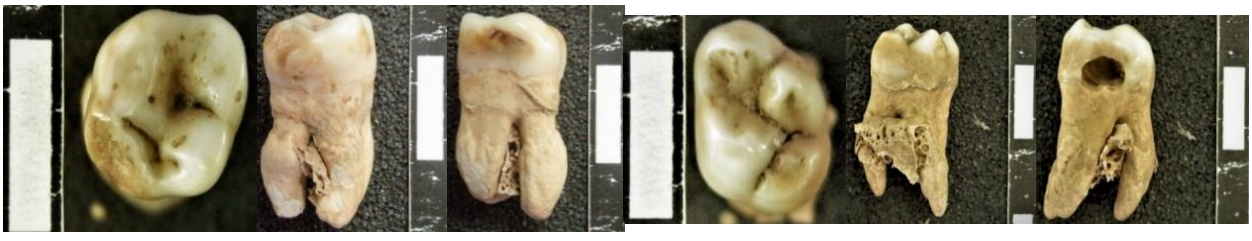


a b

Figure 42: Occlusal and buccal views of a) SP 7544, b) SP 7568

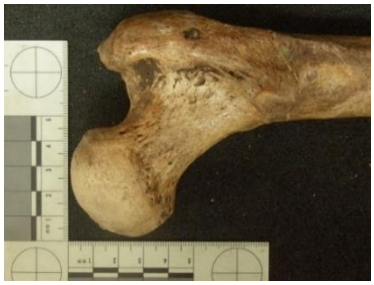
a b

Figure 43: Occlusal and buccal views of a) SP 7535 b) SP 7581



a b

Figure 44: Occlusal and buccal views of a) SP 7575 b) SP 7579



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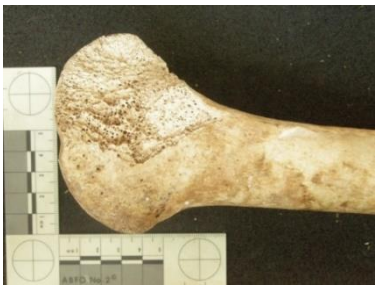

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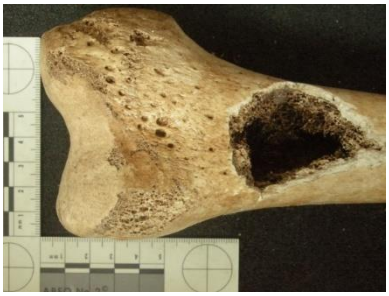
c

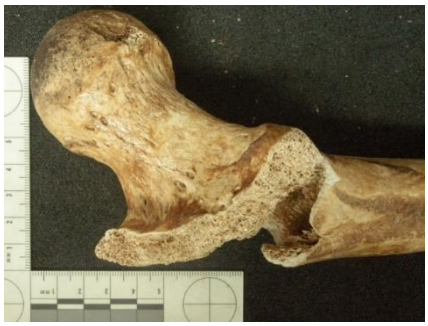


d

e

f

g



h

Figure 45: Sample pictures for bones after sample extraction a) SP 7533b, b) SP 7525, c) SP 7568, d) SP 7535, e) SP 7544, f) SP 7579, g) SP 7581, h) SP 7575

a b

c d

e f

g h

Figure 46: The femur bones after sample extraction from sample a) SP 7579, b) SP 7535, c) SP 7525, d) SP 7544, e) SP 7533b, f) SP 7581, g) SP 7568, h) SP 7575

Appendix II

Table 9: Additional elemental comparison with previously published values for tooth enamel.

Element	Present study (median)	Range	Chaudhri and Ainsworth, 1981 (contemporary, South Australia)	Frank et al., 1989 (contemporary, France)	Cleymaet et al., 1991 (contemporary, Belgium)	Carvalho et al., 1998 (contemporary)
Mn	5.57	1.3-17	5-25			
Fe	26.67	10.8-45.4	15-100		0-157	3.4-13
Cu	0.76	0.00-8.7	10-30		0-30	0-2.8
Zn	98.73	4-256	50-150	20-450	9.9-806	124-332
Sr	279.75	200-664	100-150	130-280	13-1400	54-140
Ba	10.21	5.1-16.6				
Pb	0.24	0.1-0.45			0-156	

All values in ppm.

Table 23: Trace Elemental results (ppm) for tooth enamel samples.

Sample	P	Ca	Cu	Fe	Mn	Zn	Pb	Sr	Ba	Ca/P
7525	180765.09	358116.13	0.22	30.29	2.39	159.55	0.29	238.79	6.33	1.98
7533 b	149662.55	548190.06	0.41	32.32	2.61	112.56	0.23	283.21	11.40	3.66
7535	159525.16	308380.63	0.76	42.93	7.30	106.39	0.44	403.71	16.25	1.93
7544	151518.79	373728.52	<0.00	15.68	2.59	272.85	0.43	279.75	5.91	2.47
7568	164162.96	304542.22	5.04	57.95	6.02	15.78	0.26	660.21	12.01	1.86
7575	157648.70	332167.07	0.05	12.93	6.38	30.10	0.26	210.10	8.69	2.11
7579	161905.28	310120.57	0.79	24.76	16.53	113.48	0.13	199.90	10.62	1.92
7581	164085.03	317681.29	<0.00	10.74	1.66	82.06	0.10	593.40	7.58	1.94

Table 24: Trace Elemental results (ppm) for trabecular bone samples.

Sample	P	Ca	Ba	Mn	Pb	Cu	Fe	Sr	Zn	Ca/P
SP 7525	127475.76	272215.12	62.82	5.60	1.28	5.98	267.09	1096.38	145.97	2.14
SP 7533 b	102020.90	225342.45	141.79	158.27	121.17	28.91	5019.54	1399.66	179.17	2.21
SP 7535	113845.81	259021.61	49.66	21.76	1.47	5.65	411.48	877.15	116.83	2.28
SP 7544	125504.44	276125.96	101.53	7.44	1.16	9.59	457.48	978.20	168.88	2.20
SP 7568	97802.03	244246.21	168.43	217.56	118.51	38.37	4566.05	1244.56	168.18	2.50
SP 7575	98020.27	241348.60	133.27	286.10	106.77	30.60	7019.96	1067.48	139.55	2.46
SP 7579	144025.02	313074.67	50.67	5.12	1.02	3.26	203.90	1044.13	144.48	2.17
SP 7581	74171.93	174137.81	193.37	203.85	71.77	39.46	5388.18	911.22	126.57	2.35

Table 25: Trace Elemental results (ppm) for compact bone samples.

Sample	P	Ca	Ba	Sr	Mn	Fe	Cu	Pb	Zn	Ca/P
SP 7525	120995.74	254480.89	34.42	808.97	6.56	221.08	7.01	0.81	178.07	2.10
SP 7533b	138526.05	301299.59	189.26	1921.27	9.32	89.20	11.02	0.88	151.94	2.18
SP 7535	135749.35	321290.59	56.47	1168.00	1.91	32.14	6.32	0.80	103.96	2.37
SP 7544	141407.63	312464.82	67.74	1036.17	1.20	22.70	2.76	0.54	144.62	2.21
SP 7568	131788.46	299133.84	269.01	2003.85	2.74	26.19	17.05	0.23	116.16	2.27
SP 7575	144490.96	323791.88	552.16	2073.78	9.41	33.73	16.03	0.14	108.31	2.24
SP 7579	147382.56	314813.19	33.11	1262.89	1.05	28.37	3.03	0.29	110.02	2.14
SP 7581	135489.26	295369.54	101.70	1176.08	12.98	389.50	9.99	0.39	111.79	2.18

Table 26: Variance in the results for concentration of each element.

Variance	P	Ca	Ba	Sr	Mn	Fe	Cu	Pb	Zn
Tooth enamel	$3.0*10^{8}$	$1.8*10^{10}$	1.1*10	$3.1*10^{4}$	2.3*10	$4.1*10^{2}$	4.9	$1.3*10^{-2}$	$5.1*10^{3}$
Compact bones	$6.8*10^{7}$	$6.2*10^{8}$	$2.9*10^{4}$	$2.3*10^{5}$	2.0*10	$1.7*10^{4}$	2.7*10	$7.9*10^{-2}$	$9.1*10^{2}$
Spongy bones	$4.5*10^{8}$	$1.6*10^{9}$	$2.9*10^{3}$	$3.0*10^{4}$	$1.3*10^{4}$	$7.6*10^{6}$	$2.3*10^{2}$	$3.1*10^{3}$	$5.1*10^{2}$

Table 27: log Ba/Sr, log Ba/Ca, log Sr/Ca, Zn/Ca values for cortical bones.

Samples	Zn/Ca	log (Ba/Sr)	log (Sr/Ca)	log (Sr/Ca)*	log (Ba/Ca)	log (Ba/Ca)*
SP 7525	0.70	-1.37	-2.50	0.50	-3.87	-0.87
SP 7533b	0.50	-1.01	-2.20	0.80	-3.20	-0.20
SP 7535	0.32	-1.32	-2.44	0.56	-3.76	-0.76
SP 7544	0.46	-1.18	-2.48	0.52	-3.66	-0.66
SP 7568	0.39	-0.87	-2.17	0.83	-3.05	-0.05
SP 7575	0.33	-0.57	-2.19	0.81	-2.77	0.23
SP 7579	0.35	-1.58	-2.40	0.60	-3.98	-0.98
SP 7581	0.38	-1.06	-2.40	0.60	-3.46	-0.46

All ratios in (ppm/ppm)

*ratios in (ppm/mg/g)

Appendix III

Table 11: Table showing t-test results for Sr in teeth enamel in males and females.

t-Test: Two-Sample Assuming Unequal Variances		
	Females	Males
Mean	233.002	484.267
Variance	1391.604	30393.48
Observations	4	4
Hypothesized Mean Difference	0	
Df	6	
t Stat	2.818716	
t Critical two-tail	2.447	
Alpha	0.05	

Table 13: Table showing t-test results for Pb in teeth enamel among samples from church nave and cloister courtyard.

t-Test: Two-Sample Assuming Unequal Variances		
	Church	*Cloister*
Mean	0.385714	0.194781
Variance	0.00722	0.005326
Observations	3	5
Hypothesized Mean Difference	0	
df	6	
t Stat	3.240333	
P(T<=t) two-tail	0.031664	
t Critical two-tail	2.447	

Table 15: Example of ANOVA for Mn. F value > Fcritical which leads to the rejection of null hypothesis that all the means are equal. $p < 0.05$.

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Teeth	16	90.98	5.68	22.34		
compact	16	90.35	5.65	19.59		
spongy	16	1811.43	113.21	12602.59		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Between Groups	123376.6	2	61688.31	14.63598	1.27E-05	3.204317
Within Groups	189667.8	45	4214.84			
Total	313044.4	47				

Table 16: ANOVA for Zn showing the difference between the means is not significant. F<Fcritical, P>0.05.

Anova: Single Factor						
SUMMARY						
Groups	Count	Sum	Average	Variance		
Teeth	16	1694.87	105.93	5851.32		
compact	16	1951.25	121.95	810.04		
spongy	16	2271.86	141.99	484.89		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	10446.82	2	5223.41	2.192788	0.12339	3.204317
Within Groups	107193.9	45	2382.08			
Total	117640.7	47				

Table 18: Tukey's HSD results at confidence interval of 95%age.

a) Iron

	Tukey's Honestly Significant Difference			**HSD**		
		critcal q (α, r, dfW)				
	xi-xj	critical q (0.05, 3, 45)	standardized error	95%age conf Interval for μi-μj		Significant at 0.05?
teeth-compact	-76.06	3.4275	403.13	-1457.79	1305.66	
teeth-spongy	-2887.4	3.4275	403.13	-4269.13	-1505.68	Yes
compact-spongy	-2811.35	3.4275	403.13	-4193.07	-1429.62	Yes

b) Copper

	Tukey's Honestly Significant Difference	**HSD**		

		critcal q (α, r, dfW)				
	xi-xj	critical q (0.05, 3, 45)	standardized error	95%age conf Interval for μi-μj		Significant at 0.05?
teeth-compact	-8.13	3.4275	2.36	0.030	-16.23	
teeth-spongy	-19.20	3.4275	2.36	-11.10	-27.31	Yes
compact-spongy	-11.07	3.4275	2.36	-2.97	-19.18	Yes

c) Manganese

	Tukey's Honestly Significant Difference			**HSD**			
		critcal q (α, r, dfW)					
	xi-xj	critical q (0.05, 3, 45)	standardized error	95%age conf Interval for μi-μj		Significant at 0.05?	
teeth-compact	0.039	3.4275	16.23	55.67	-55.59		
teeth-spongy	-107.53	3.4275	16.23	-51.89	-163.16	Yes	
compact-spongy	-107.57	3.4275	16.23	-51.94	-163.20	Yes	

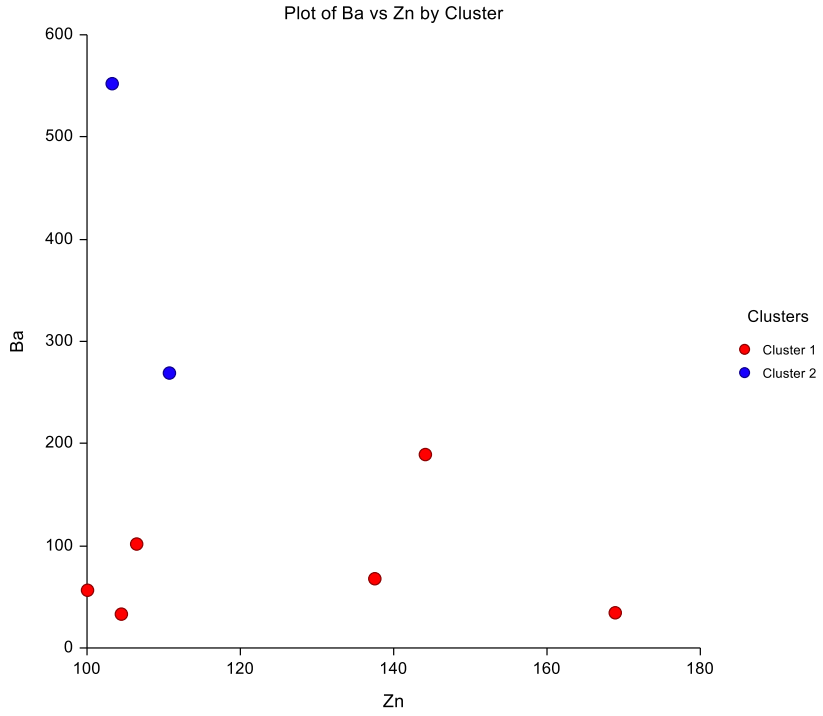
d) Barium

	Tukey's Honestly Significant Difference			**HSD**			
		critcal q (α, r, dfW)					
	xi-xj	critical q (0.05, 3, 45)	standardized error	95%age conf Interval for μi-μj		Significant at 0.05?	
teeth-compact	-153.14	3.4275	25.97	-64.11	-242.16	Yes	
teeth-spongy	-102.84	3.4275	25.97	-13.81	-191.87	Yes	
compact-spongy	50.29	3.4275	25.97	139.32	-38.73		

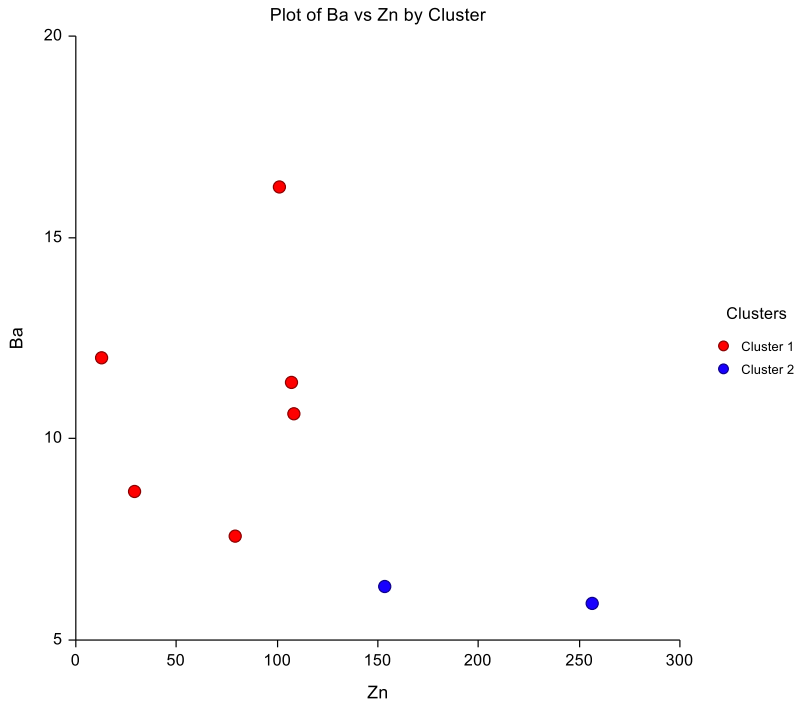
e) Lead

	Tukey's Honestly Significant Difference			**HSD**			
		critcal q (α, r, dfW)					
	xi-xj	critical q (0.05, 3, 45)	standardized error	95%age conf Interval for μi-μj		Significant at 0.05?	
teeth-compact	-0.24	3.4275	7.99	27.13	-27.62		
teeth-spongy	-52.63	3.4275	7.99	-25.25	-80.00	Yes	
compact-spongy	-52.38	3.4275	7.99	-25.00	-79.76	Yes	

Appendix IV



a



b

Figure 47: Bivariate plot between Ba (ppm) and Zn (ppm) for a) compact bone b) tooth enamel